ADJUVANT STUDIES OF CYTOKINES IN DNA VACCINE AGAINST VIRAL HAEMORRHAGIC SEPTICEMIA VIRUS (VHSV) OF JAPANESE FLOUNDER, *Paralichthys olivaceus*

A THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

TOKYO UNIVERSITY OF FISHERIES

BY

EMMADI DHANWANTHARI

2004

LABORATORY OF GENETICS AND BIOCHEMISTRY

TOKYO UNIVERSITY OF FISHERIES

Declaration

I hereby declare that this thesis has been composed by myself and is a result of my own investigations. It has neither been accepted, nor submitted for any other degrees. All sources of information have been duly acknowledged.

Emmadi Dhanwanthari

CONTENTS

Adjuvant studies of Cytokines in DNA vaccine against viral haemorrhagic septicemia virus (VHSV) of Japanese flounder, *Paralichthys olivaceus*

Emmadi Dhanwanthari
Laboratory of Genetics and Biochemistry
Tokyo University of Fisheries

ABSTRACT

Cytokines are low molecular weight soluble proteins secreted by the cells of the innate and adaptive immunity that mediate many of the functions of these cells. They are often pleotropic and redundant. Viral hemorrhagic septicemia is an infectious viral disease of fish, caused by viral hemorrhagic septicemia virus (VHSV) and is responsible for economic losses in aquaculture farms in Japan and around the world. DNA vaccine has been developed for VHSV, but to sustain the immune response of this DNA vaccine for a long time adjuvant studies are required. In the present study we described the adjuvant studies of IL-1β, IL-8 and JFCC1 using VHSV as a model system. We also enumerated the gene expression profile of immune related genes of Japanese flounder kidney cells induced by IL-1β, IL-8 and JFCC1.

The full length genes of IL-1β, IL-8 and JFCC1 of Japanese flounder were cloned into the CMV driven DNA vaccine expression vector. VHS glycoprotein DNA was also cloned into expression vector. The plasmids were extracted in a large scale. The fish were co-administered by intra/muscular injection in 0.1 μg of VHSV glycoprotein along with 10 μg each of IL-1β, IL-8 and JFCC1 respectively. After 8 days post-vaccination, the fish were challenged with $1X10^3$ TCID 50 VHS virus intra/peritoneally. The cumulative

mortalities and RPS values were recorded to study the adjuvant effects. For cDNA microarray analysis, 10 μg each of IL-1β, IL-8, JFCC1 was injected into Japanese flounder fish and kidney was sampled at 1 day, 3 day and 7 day. cDNA was labeled with Cy3 and Cy5, hybridized to the microarray chip and the results were analyzed.

Fourty eight percent mortality was observed in 0.1 glycoprotein vaccine group against 23-30 percent in cytokine adjuvant groups. The relative percentage survival was significantly high in 0.1 μg VHS g along with 10 μg of adjuvants, compared with fish given the glycoprotein DNA vaccine alone. At least 18 % increase in the efficiency of the DNA vaccine was observed with adjuvants.

Of 871 cDNAs on a microarray, 93 genes (10.7%) were up-regulated or down-regulated by IL-1β at 1, 3 and 7 days post injection. The induced up-regulated genes were highest on 1 day followed by 3 day and 7 day. Seven percent of known and 3.7% of unknown genes of the 871 tested genes were differentially expressed. Of the genes tested, 7.4% were up-regulated and 3.3% were down regulated. Cytokine genes such as tumor necrosis factor, G-CSF and chemokine receptor A were induced in response to IL-1β. Cell surface antigens such as IgM, MHC class I and CD20 receptor were up-regulated. Signal transduction genes such as Toll-like receptor 1 and SH3P2 were also up-regulated. The glucocorticoid receptor and cAMP early repressor were down regulated in our microarray analysis.

The induced genes were high on 1day followed by 3 day and 7 day by IL-8 injection. On the contrary, the induced gene expression was high on 3 day followed by 1 day and 7 day by JFCC1. Of the genes tested, 3.6% were up-regulated and 1.6% were down-regulated by IL-8. JFCC1 up-regulated 6.1% and down-regulated 1% of the total tested genes. The important genes that up-regulated were Ig M, MHC class I , T cell receptor β , Ig D and heat shock factor binding protein by CXC chemokine. Whereas the JFCC1 up-regulated CXC chemokine, CC chemokine receptor, Ig M, lysozyme and amyloid protein.

In conclusion, IL-1β, IL-8 and JFCC1 showed adjuvant effects in DNA vaccine against VHSV in our study. All these three genes are strong mediators of innate immunity, humoral immunity and inflammation processes. IL-1β, IL-8 and JFCC1 exhibited cellular immunity. IL-1β alone exhibited the signal transduction inducer property. Still further studies are needed especially in the quantification of dosage and duration of immunity to assess adjuvant properties of these cytokine genes.

Chapter 1

Introduction

1.1. AQUACULTURE TREND IN 21st CENTURY: Fish provide a significant source of animal protein. Global production from capture fisheries and aquaculture is very significant for global food security, providing more than 15 percent of total animal protein supplies. In 2001 total fishery production excluding aquatic plants was reported to be 130.2 million tonnes, of which 37.9 million tonnes came from aquaculture practices.[1] Aquaculture production, excluding aquatic plants, reached 37.9 million tonnes by weight and 55.7 billion dollars by value in 2001. In 2001, more than 80% of the total world import value was concentrated in the developed countries. Japan was the major importer accounting for 23% of the total value.[1] According to FAO,[2] the projections of world fishery production in 2010 range between 107 and 144 million tonnes. Estimated quantities which will be available for human consumption range between 74 million tonnes and 114 million tonnes. Most of the increase in fish production is expected to come from aquaculture which is growing rapidly. According to FAO statistics, aquaculture is growing more rapidly than all other animal food producing sectors. Worldwide, the sector has increased at an average compounded rate of 9.2 percent per year since 1970, compared with only 1.4 percent for capture fisheries and 2.8 percent for terrestrial farmed meat production systems.

1.1.1. Japanese flounder culture: Japanese flounder, *Paralichthys olivaceus* is a temperate marine flat fish that belongs to the family *Paralichthyidae*, Order *Pleuronectiformes*, and class *Actinopterygii*. Its common names are bastard halibut, olive flounder, Japanese flounder and hirame. Its habitat ranges from the western Pacific from Japan, Kurile Islands and the Korea peninsula to the South China Sea. Japanese flounder culture was initiated in Japan in the 1980s and widely introduced to Korea and China

during the latter half of 1980s. In 2001, Japanese flounder production ranked fourth in overall marine fish culture in Japan. Its production was estimated at 11 billion yen, which corresponds to 4.8% of a total marine fish production of 230.5 billion yen (Gross Fisheries Output, 2001, the ministry of agriculture, forestry and fisheries of Japan– www.maff.go.jp/esokuhou/2002_f2.pdf). Japanese flounder is a temperate marine species and grows fastest at 15 to 25 ^{0}C. It is usually cultured in land-based tanks in which the water temperature can be controlled.

1.1.2. Viral diseases in Japanese flounder: Aquaculture production is mainly affected by the incidence of diseases originating from viral, bacterial and parasitic origin.[3-4] It has been estimated that ten percent of the all cultured aquatic animals are lost because of infectious diseases alone. Japanese flounder is also susceptible to many viral and bacterial diseases. Some of the important viral diseases that have been identified in Japanese flounder are hirame rhabdovirus, viral hemorrhagic septicemia, viral nervous necrosis and viral ascites caused by birna virus.

Viral hemorrhagic septicemia (VHS) is an infectious viral disease that causes serious economic problems in fish farms. This has been identified in Japanese flounder in Japan. The virus is placed within the family *Rhabdoviridae* and in the *Novirhabdovirus* genus. VHS generally occurs at a temperature range of 4-14 ^{0}C. The disease is characterized by high mortality, with the affected fish being dark, lethargic and showing haemorrhages at the base of fins and in the gills. Dead fish at this stage often have massive haemorrhages in the abdominal cavity. The second stage is one of chronic disease, where the predominant feature is a very black coloration, and anemia. The gills are very pale and exophthalmos is a common feature. The third clinical stage, from which

the virus is not usually isolated, is associated with the cessation of mortality, but affected fish show darkness and exophthalmos, but the main feature is swelling and discoloration of the kidney (www.fishpathogens.net). The viral genome is composed of an approximately 11-kb single-stranded RNA. It contains six genes in the order 3`-N-P-M-G-NV-L-5`, which encode five structural proteins such as a nucleocapsid protein, polymerase associated protein, matrix protein, glycoprotein, non-structural protein and RNA dependent polymerase (www.fishpathogens.net).

Chemicals and antibiotics used for disease control have had undesirable side effects such as accumulation in the flesh of animals, development of drug resistance, and contamination of the aquatic environment.[4] Researchers have shifted their focus to prevention of diseases, in particular vaccine development, to reduce the possibility of infections. The definite need to produce effective vaccines against a number of viral and bacterial diseases has led to a better understanding of fish immunity and the factors that influence its function.

1.2. IMMUNITY IN FISHES: In general, the immune response of animal species is of two types: innate immunity and adaptive immunity. Fish immunity exhibits similarities to the immunity other vertebrates and can be classified into these two general categories.[5] Innate immunity consists of mechanisms that exist before infection. It is capable of rapid responses to microbes, and reacts in essentially the same way to repeated infections.[6] The innate or non-specific immunity provides early lines of defense against inflammation, especially from microbial or pathogenic agents including ecological toxins and parasites breaching through the epithelial barrier.[7-8] These barriers include skin, mucus, gill and a variety of cell types or substances. Invertebrates also possess an innate immune system.[5]

In contrast, adaptive or acquired immunity is more specific to the pathogens and responds to repeated exposures to the same microbe. The jawed fishes from Chondrichthyes to Osteichthyes clearly show activities or pathways of adaptive immunity, including the complement pathway.[5] This immunity involves antigenic activation of specific reactive lymphocytes. Following antigen recognition, lymphocyte populations change, resulting in lymphocyte proliferation, antibody production and immunological memory induction.[9] Immune responses require the interaction of multiple cell types and mediators. Both innate and adaptive immunities require cytokines which regulate the communication between antigen-presenting cells (APCs), lymphocytes and other host cells in the course of an immune response.[10]

1.2.1. Cytokines as a link between innate and adaptive immunity: Cytokines are proteins secreted by the cells of innate and adaptive immunity that mediate many of the functions of these cells.[6] Cytokines are produced in response to microbes and other antigens, and different cytokines stimulate diverse responses of cells involved in immunity and inflammation. In the activation phase of immune responses, cytokines stimulate the growth and differentiation of lymphocytes, and in the effector phases of innate and adaptive immunity, they activate different effector cells to eliminate microbes and other antigens. Cytokines also stimulate the development of hematopoietic cells.[6] A generally accepted functional definition of cytokines distinguishes between type-1 and type-2 cytokines. Type-1 cytokines such as IL-2, IL-12, IL-15, IFN-γ, IFN-α and IFN-β are involved in T helper 1 (Th-1) immune responses and induce cell mediated immunity. In contrast, type-2 cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 are involved in

Th2 immune responses and promote humoral immunity and immune deviation to a non-protective response.[10]

Different cytokines are involved in different functions. IL-2,[11] IL-4[12] and TGFβ[13] are involved in cell proliferation. IL-7[14] and IL-15[15] play important roles in cell survival. IL-12[16] is important for cell differentiation. GM-CSF, IFNα and IL-10[10] are important in antigen presentation. Some cytokines are also involved in immune cell development, such as IL-7,[17] SCF,[18] SDF[19] and IL-15[20] or act on lymphoid organs, such as LT and TNF.[21] In general, acute and chronic inflammations are induced and affected by cytokines such as IL-1, TNFα, IL-6, IL-12, GM-CSF, chemokines and IFNγ. These cytokines are called proinflammatory cytokines. Another cytokine group, which acts in part by reducing the production of proinflammatory cytokines, is termed anti-inflammatory. Members of this group such as IL-10, IL-4, IL-13 and TGF-β, can reduce the production of IL-1, IL-12, IFNα and TNFα from macrophage.

1.2.2. Cytokine genes in fish: Secombes *et al.*[22-23] reviewed cytokine genes that have been identified in recent years. An inflammatory insult will result in a cytokine cascade where tumor necrosis factor is released, followed by interleukin-1β. Recently TNF-α sequences were published for Japanese flounder,[24] rainbow trout[25] and channel catfish.[26] Zou *et al.*[27] reported two isoforms of TNF in rainbow trout. TGF-β is a pleotropic cytokine involved in tissue remodeling, wound repair, development and haematopoiesis, and is expressed in wide range of cells and tissues. A full-length of cDNA has been sequenced in rainbow trout, and translates into a 382 amino acid precursor molecule[22] TGF-β1 has been identified in carp[28] and sea bream.[29] Interleukin-1β and other chemokines were also studied in fishes.

1.2.3. Interleukin-1β

Evolution: Interleukin-1β (IL-1β) is a member of the IL-1 cytokine family having a β-trefoil structure composed of 12-β sheets,[30] and plays a pivotal role in the inflammatory response as well as in the maturation and proliferation of many immune cell types.[31] Its recent discovery in teleost fish dates cytokine evolution at c.350 ago.[32] The intron-exon organization of the three IL-1 genes (IL-1α, IL-1β and IL-1Ra) suggests duplication of a common gene some 350 million years ago. Before this common IL-1 gene, there may have been an ancestral gene from which fibroblast growth factor (FGF) also evolved, since IL-1 and FGF share significant amino acid homologies, lack a signal peptide and form an all β-pleated sheet tertiary structure.[31] IL-1β is synthesized by activated monocytes and macrophages as a precursor with little activity.

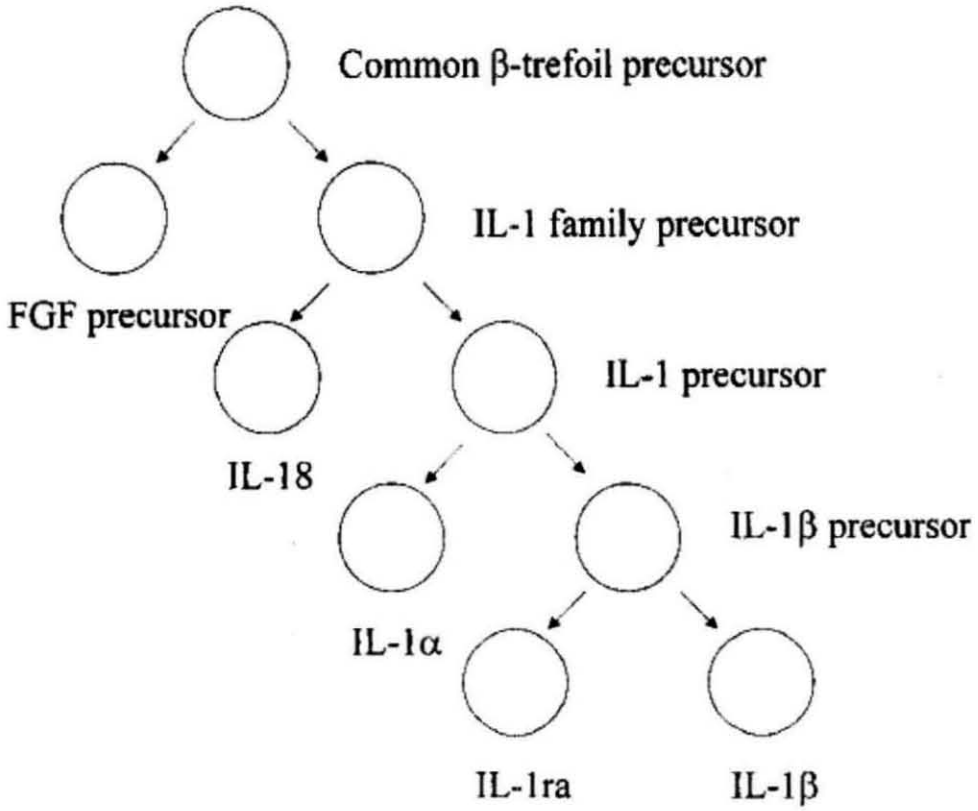


Fig.1 IL-1 Family (Secombes *et al.*, 1999)[32]

Structure: The genomic DNA and cDNA of many species have been cloned in mammals and fish. In fishes the cDNA has been cloned and sequenced in rainbow trout, [33] carp,[34] seabass,[35] and seabream.[36] The translated open reading frames gave a predicted peptide with molecular weights ranging from 28-31 kD for different fish species. The precursor peptide doesn't have a biological activity in mammals. The precursor is then processed by caspase-1, also called interleukin-1-converting enzyme (ICE), releasing a mature active peptide.[37] The cleavage of proIL-1β at the aspartic acid alanine positions (amino acids 116-117) is a property of a specific intracellular cysteine protease known as ICE.[38] The aspartic acid at position 116 of the pro-IL-1β is the recognition amino acid for ICE cleavage. Prevention of the cleavage the ICE precursor may also account for apparent inhibition of IL-1β synthesis. ICEα cleaves the the ICE precursor and pro IL-1β. It is presumed that ICEβ and γ also process precursor ICE. ICE activity and IL-1β secretion also regulated by events associated with inflammation.[39] Mammalian IL-1β precursor is cleaved by ICE at Asp-X bound (where X is normally a small hydrophobic residue) to yield a 17 kDa carboxyl terminus derived mature polypeptide which is then transported out of the cells, supporting the hypothesis that IL-1β post-translational processing involves a commitment to cell death.[37] The gene ced-3 in the nematode, *Caenorhabditis elegans*, codes for a protein homologous to human ICE.[40] During embryonic development of the worm, this gene is expressed in specialized cells and is thought to be responsible for programmed cell death.

Surprisingly, in teleosts the IL-1β precursor sequenced to date lacked the interleukin-1-converting enzyme recognition site.[35] Despite this difference from

mammalian IL-1β, the similarity in secondary structure is high.[32] It is a common feature to have ATTTA motif in the 3' UTR of all inflammatory molecules.[41] Almost all teleosts IL-1βs cloned to date possess this ATTTA motifs in their 3' untranslating regions, suggesting that their mRNA expression is tightly regulated. The teleosts IL-1β show high homology among different fish species. Japanese flounder has highest homology with seabass IL-1β. The amino acid regions that form the secondary structure of 12 β-sheets were the areas showing the highest homology. The organization of the teleosts IL-1βs is different from that of mammals. Mammalian and carp IL-1β genes contain 7 exons[42] whereas the trout sequence consists of 6 exons.[43] However the organization of the seabream IL-1β is different to those of mammals and fish and comprises only five exons.[36]

Gene expression: In human monocytes, IL-1β mRNA levels rise rapidly within 15 min but depending on the stimulant, start to fall after 4h. The decrease is due to the synthesis of a transcriptional repressor and a decrease in mRNA life.[44] Using IL-1 itself as a stimulant of its own gene expression, IL-1β mRNA levels are sustained for over 24 h.[45] Raising cAMP levels in monocytes with histamine enhances IL-1β gene expression and protein synthesis.[46] Inhibition of translation by cyclohexamide results in enhanced splicing of exons, excision of introns and increased levels of mature mRNA. Thus synthesis of mature IL-1β mRNA requires an activation step to overcome an apparently intrinsic inhibition to process precursor mRNA.[44] Dissociation between transcription and translation is the main characteristic feature of IL-1β[47] and also of TNFα.[48] Even though there are vigorous signals for transcription by a variety of agents such as C5a, most of the IL-1β is degraded and no significant translation takes place and there is little significant

elongation of the peptide although the IL-1β mRNA assembles into large poly ribosomes.[39] However, adding of bacterial endotoxin or IL-1 itself to cells with high levels of steady state mRNA results in augmented translation.[48] One possible explanation is that stabilization of the AU-rich 3' untranslated region takes place in cells stimulated with LPS. These AU-rich sequences are known to suppress normal hemoglobin synthesis. Another explanation is that IL-1 stabilizes its own mRNA by preventing deadenylation, as it does for chemokine gro-α.[39] Removal of IL-1 from cells after 2 h increases the shortening of poly (A) and IL-1 apparently is an important regulator of *gro* synthesis because it prevents deadenylation. Of the several cytokines induced by IL-1, large amounts of the chemokine family are produced in response to low concentrations of IL-1. Following synthesis, pro IL-1β remains primarily cytosolic until it is cleaved and transported out of the cell. The IL-1β propiece (amino acids 1-116) is myristoylated on lysine residues.[49] Pro IL-1β has no known membrane form and proIL-1β is only marginally active.[50] Release of the mature IL-1β appears to be linked to cleavage of the aspartic acid-alanine peptide cleavage by the ICE.[39]

Usage of endotoxin or other microbial stimuli, and prostaglandins and prostacyclin had little effect on transcription but reduced translation of IL-1.[51] Blocking cyclooxigenase can increase production of IL-1, particularly when cells are stimulated by agents that stimulate large amounts of PGE. PGE-induced suppression of IL-1 translation appears to be via the induction of cAMP. The addition of PGE or dibutyryl cAMP suppresses IL-1 synthesis.[51] Histamine binding to the type-II histamine receptor raises cAMP levels and histamine reduces LPS induced IL-1 synthesis in human monocytes with little effect of IL-1 gene expression.[52] The effects of cAMP on IL-1 synthesis and

gene expression are dependent on the type of stimulant and the conditions of cell culture. Histamine, which reduces LPS-induced IL-1 synthesis via a cAMP pathway, enhances IL-1-induced IL-1 synthesis and gene expression.[51] Similarly, PGE_2 enhances IL-1 induced IL-1 and IL-1-induced IL-1 is reduced by cyclooxigenase inhibitors.[51] Control of IL-1 transcription and translation is affected by other cytokines. IL-1 is a stimulus of its own gene expression and synthesis in blood mononuclear, fibroblasts, endothelial and smooth muscle cells.[53] TNF,[54] granulocyte-macrophage colony stimulating factor (GM-CSF),[51] and macrophage-CSF (M-CSF) also stimulate IL-1 production. IL-1 transcription is suppressed by IL-4,[55] IL-6[56] and transforming growth factor β (TGF-β).[51] In general, corticosteroids inhibit the transcription of IL-1, TNF and nearly all cytokines. In human volunteers injected with corticosteroids just before an intravenous injection of endotoxin, there are reduced levels of circulating IL-1β, TNF and IL-6.[39] These reductions in IL-1β, TNF and IL-6 take place with out suppressing IL-1Ra production.[39] Increased synthesis of IκB with decreased translocation of NFκB is thought to account for the suppressive effect of glucocorticoids on cytokine synthesis.[57]

Gene expression in Fish: In teleosts the expression of IL-1β was studied thoroughly. In rainbow trout there is no constitutive expression of IL-1β in lymphoid organs.[43] However, challenge for 48 h with an attenuated Gram negative bacterial pathogen *Aromonas salmonicida* [58] can induce IL-1β transcription in a range of tissues, including blood, gill, kidney, liver and spleen, but not brain.[43] Similarly, stimulation of kidney leucocytes in vitro with LPS or PHA has been shown to induce IL-1β expression.[32] A number of factors have a pronounced effect on IL-1β mRNA levels in rainbow trout head kidney leucocytes. LPS was used as an inducer of trout IL-1β mRNA, since it is the

classical stimulant of IL-1 expression in many animal models.[31] Trout leucocytes can respond to levels of LPS as low as 0.1μg/ml, although clearly this is a sub-optimal stimulation of IL-1β expression. Maximal stimulation was seen using doses of LPS> 1.5 μg/ml. This is much higher than the doses of LPS required for stimulation of IL-1β expression in mammalian monocytes, where concentrations as low as 10-20 pg/ml are sufficient.[51] The expression occurs quickly following stimulation with LPS, with transcripts being detectable by 1 h post-stimulation. Peak transcript levels appeared to occur between 2.5 and 4 h post-stimulation. Transcript levels were still high at 8 h post-stimulation but then declined markedly by 24 h and were not detectable by 72 h. The rapid decline in IL-1β transcript following stimulation with LPS has been attributed to the synthesis of a transcriptional repressor and unstable mRNA due to the presence of AUUUA motifs in the 3' UTR[31] which are also present in the trout transcript. IL-1β expression in cultured trout head kidney leucocytes was demonstrated with respect to temperature.[59] They reported that the temperature appears to be a relatively quick event, in that cells cultured at 14 ^{0}C and then transferred to 4 ^{0}C or 22 ^{0}C at the time of stimulation with LPS show an impact of changing temperature relative to cells kept at 14 ^{0}C. However, transfer to 22 ^{0}C at the time of stimulation did not result in as large an effect on IL-1β expression as seen in cells cultured for 5 days at 22 ^{0}C prior to stimulation. In contrast, when cells cultured at 14 ^{0}C were transferred to 4 ^{0}C at the time of LPS stimulation there was a more marked inhibition of IL-1β expression relative to cells cultured at 4 ^{0}C for 5 days prior to stimulation. It is clear from the above observation that low temperatures markedly inhibit the transcription of the IL-1β gene in trout leucocytes and this is likely to lead to poor IL-1β protein production. Cortisol was also

shown to inhibit IL-1β transcription by trout head kidney leucocytes.[59] Engelsma *et al.*[42] studied the in *vivo* expression of carp IL-1β mRNA in multiple organs. They found a predominant expression in the immune organs head kidney and spleen. In carp, addition of PMA or LPS *in vitro* resulted in expression of both intron-containing and fully spliced IL-1β RNA products within 1 h. Expression of intron-containing products was seen until 3 h after LPS stimulation and fully spliced products exceeded 8 h after stimulation with a peak at 2-3 h. The NF-κB inhibitor, PDTC, was shown to completely suppress the LPS-induced expression, clearly indicating that the NF-κB transcription factor is involved in the LPS-induced expression of carp IL-1β.[42] Jarrous and Kaempfer[44] have described the retinoic acid (RA)-induced IL-1β expression in human peripheral blood mononuclear cells. RA induces the accumulation of precursor transcripts but fails to yield mature RNA. In carp similar expression pattern was observed.[42] Tucker *et al.*[60] reported detection of bombarded CAT (reporter gene) expression in fish of the CMV promoter group and fish of the SV-40 promoter group after plasmid DNA injection. The CAT expression in the fish group injected with CMV promoter was higher than that in the fish group injected with SV-40 promoter. Kono *et al.*[61] studied the IL-1β protein expression in serum on 1, 3 and 5 days after pCMV-IL-1β plasmid injection.

1.2.4. Chemokines

Chemokines (chemoattractant cytokines) are a complex super family of small (6-14 kDa) secreted proteins that were initially characterized through their chemotactic effects on a variety of leucocytes.[62] To date, at least 40 distinct chemokines have been well characterized. Chemokines have a plethora of overlapping functions and are produced by a variety of cell types during an inflammatory response. Generally, chemokines act on

more than one type of leucocyte, and *in vitro* responses include chemotaxis, enzyme release from intracellular stores, oxygen radical formation, and shape change through cytoskeletal rearrangement, generation of lipid mediators and induction of adhesion to endothelium or extracellular-matrix proteins.[62] Lymphocyte traffic is important in regulation of the immune system. Apart from lymphocyte migration, chemokines also act on angiogenesis/angiostasis, lymphoid organ development, cell recruitment, Th1/Th2 development and metastasis.[63] Chemokines within a particular group show closely similar structures, with sequence homologies ranging from 20% to 90%. Chemokine domains are defined by the presence of four cysteines in highly conserved positions. One major chemokine subfamily is called 'CXC' because the two amino acids nearest the N-termini of these proteins are separated by a single amino acid. This is in contrast to another subfamily which is called 'CC' because these cysteines are adjacent.[64] CX_3C or δ chemokines form a unique chemokine group, exhibiting structural distinctions not found in other chemokines. Three amino acids interrupt the first and second cysteines forming a mucin-like stalk and both transmembrane and cytoplasmic domains.[65] C (γ) chemokines, the remaining subfamily of chemokines, lack the second cysteine, yet are functional.[66]

Evolution: Amino acid sequence analysis of CXC and CC chemokines indicate that the mammalian and primitive vertebrate chemokines diverged from the ancestral gene before the appearance of mammals.[67] CXC and CC chemokine receptors branched early from an ancestral gene before the divergence of the mammalian order and it has been suggested that they have co-evolved with chemokine ligands.[68] However, the evolutionary track of the receptor gene was possibly different from that of the chemokines.[67] Almost all CC chemokine genes and CXC chemokine genes are grouped at 17q11.2-12 and at 4q13

respectively, suggesting that chemokines arose by duplication and divergence from a primordial chemokine gene, with an early split into the two loci. Similar clustering has been observed among genes encoding chemokine receptors. Mice appear to have fewer chemokines than humans and some of these chemokines may have occurred recently in evolutionary terms.[64] Chemokines have been found in non-mammalian vertebrates such as birds and frogs. Sick *et al.*[69] reported two novel CC and CXC chemokines from a chicken macrophage cell line upon stimulation with LPS. This CC chemokine is a mammalian MIP-1β homologue and the CXC chemokine is an ortholog of mammalian human IL-8. Hughes *et al.*[70] identified three other types of CC chemokines from chicken. Their DNA sequences share identity with MIP-3α, RANTES and several CC chemokines. Phylogenetic tree analysis suggests that individual chicken and human chemokines were derived from common ancestral genes, thus reflecting their genomic positions. Lam[71] also reported a MIP-1β like compound in the supernatants of chicken leukocytes after infection with *Mycoplasma gallisepticum*. The supernatants showed chemotactic activity similar to human MIP-1β but could not be neutralized by antibody to MIP-1β, indicating more than one chemoattractant was released from chicken leukocytes. CXC chemokine in frog (*Xenopus laevis*), xSDF-1, has been reported.[72] It differs considerably in primary structure from its mammalian counterpart but, it is indistinguishable from human SDF-1 in its ability to activate both *X. laevis* CXC chemokine receptor (xCXCR4) and human CXC chemokine receptor (hCXCR4). Braun *et al.*[72] suggested the possibility that these receptors have maintained evolutionary distance between *Xenopus* and living mammals.

Structure: The primary sequence of chemokines is defined by their four invariant cysteine residues that form disulfide bonds. The amino acid sequence identities among

chemokines vary from less than 20% to over 90%. The presence of a putative signal peptide gives rise to a secreted sequence for each chemokine. A prominent secondary structural feature of chemokines is the triple-stranded antiparallel sheet that forms a sheet floor across which lies hydrophobic C-terminal helix. Proudfoot *et al.*[73] summarized that the amino acid sequences at the N-terminus preceding the first cysteine are flexible, with no specified structure. It is believed that this plays a role in activation during the process of chemokine receptor binding. The special relationship of the N-terminus, sheet and helix is maintained by two disulfide bonds. The three structural domains have distinct functions. The N-terminus is essential for the activation of G protein-coupled receptors.[74] The N-terminus and the sheet provide a scaffold for quaternary interactions resulting in high-affinity ligand binding to G protein-coupled receptors and the formation of chemokine dimers. Following the first two cysteines is a region of the structure approximately 10 amino acid residues long called the N-loop. This N-loop plays an important role in receptor binding. One strand of a 3_{10} helix follows the N-loop and is preceded by three β-strands and a C-terminal helix. The β-strands at the 30s, 40s and 50s loops, for which the numbers represent the residues of the mature protein. The secondary structure of a chemokine is formed by two disulfide bridges. The first disulfide bridge is formed by the first and third cysteines while the second bridge is arranged by the pair of second and fourth cysteines.[75] The C-terminal helix of some chemokines has been shown to interact with low-affinity glycosaminoglycan (GAGs) that are found on cell surfaces and matrix proteins.[76] The basic charge of these ligands accounts for their heparin-binding properties. The GAG-binding capabilities of the chemokines are reported to facilitate receptor interactions and the haptotactic migration of receptor-bearing cells over

matrix proteins and cellular faces. Some chemokines contain consensus sites for N-linked and/or O-linked glycosylation. The glycosylation may play a role in prolonging the chemokine half-life, and may improve GAG binding.

CXC chemokines: IL-8, NAP-2, ENA-78, GROα, GROβ, GROγ, and GCP-2 are members of the CXC subfamily. The CXC subfamily is subdivided into ELR+ and non-ELR CXC chemokines based on the presence or absence of a Glu-Leu-Arg tripeptide sequence adjacent to the CXC motif. ELR-containing CXC chemokines include IL-8, ENA-78, and GRO α, β, and γ. The non-ELR CXC chemokines include IP-10 and SDF-1.[77] IL-8 is the prototype molecule. IL-8 was originally purified as a monocyte-derived factor that attracts neutrophils, but not monocytes. IL-8 is produced by a variety of cell types including monocytes, T lymphocytes, neutrophils, fibroblasts and endothelium. IL-8 induces T lymphocyte chemotaxis and angiogenesis.[64] The IL-8 amino acid sequences have variable lengths due to extensions at the N-terminus. IL-8 is a potent neutrophil chemoattractant, which is mainly due to the presence of an ELR motif between the N-terminus and the first cysteine residue, at positions 4, 5, and 6.[78] This ELR motif is essential for high-affinity binding to CXCR2.[79] The ELR^+ CXC chemokines promote the adherence of neutrophils to endothelial cells. A gradient of chemokines promotes diapedesis and migration toward inflammatory sites, with the neutrophils associating with matrix proteins and cell surfaces.[64] Jones *et al.* [80] reported that neutrophil degranulation may be achieved more readily by IL-8-induced activation of CXCR1 than CXCR2. The ELR^+ CXC chemokines elicit chemotactic activity in endothelial cells.[81] The ELR^- CXC chemokines such as IP-10, MIG, and I-TAC, which all use CXCR3, are chemotactic for activated T cells and monocytes. Both IP-10 and MIG have potent angiostatic effects.[77]

The CXC chemokines are also reported to play roles in homeostatic development. One of the CXC chemokines, SDF-1, was identified as a pre-B cell growth-stimulating factor (PBSF). SDF-1 acts as a homeostatic hematopoietic hormone that is critical for homing and angiogenesis more so than as a proinflammatory chemokine.[82] SDF-1 is considered as a primitive chemokine due to its location on chromosome 10, whereas the other CXC chemokines are located on chromosome 4. This suggests SDF-1 probably did not arise by gene duplication with other chemokines. Furthermore, SDF-1 exhibits equidistant sequence homology to CXC and CC chemokines. Unlike other chemokines, which are highly divergent, SDF-1 is highly conserved, showing only one amino acid difference between humans and mouse.[82] SDF-1 is expressed by a wide variety of cells and tissues. It can attract a broad spectrum of target cells because its receptor (CXCR4) is widely distributed on neutrophils, monocytes, T and B cells, $CD34^+$ hematopoietic progenitor cells, bone marrow-derived dendritic cells, megakaryocytes, endothelial cells, neurons, astroglial cells, and gastrointestinal epithelial lining cells.[82]

CC chemokines: The CC chemokines generally target mononuclear cells rather than neutrophils and are either pro-inflammatory or homeostatic mediators. Based on their similarities of amino acid sequences and chromosomal location. CC chemokines are subdivided into 3 subgroups, the MCP/eotaxin subgroup, the MIP-1α/β subgroup, and the I-309 subgroup. Members of the MCP/eotaxin subgroup of CC chemokines are closely related in chromosome location, gene and protein structure and receptor utilization.[83] They attract basophils and mast cells and can induce basophil de-granulation with histamine release.[84]

A second subgroup of CC chemokines includes macrophage inflammatory protein-1 (MIP-1α), MIP-1β, RANTES and PARC. The CC chemokine subgroup of MIP-1α, MIP-1β and RANTES shows close relationships in human and mouse.[85] MIP-1α and MIP-1β share high percentage similarity in terms of amino acid and nucleotide sequences at 68% and 69%, respectively. Their high similarity is due to their co-evolution from the same primordial ancestral gene and duplication.[86] Peptidoglycans consist of heparin, heparan sulfate and chondroitin sulfate. These are located at the surface of endothelial cells or in the extracellular matrix. It is believed that the binding of chemokines to peptidoglycans establishes an immobilized gradient, which facilitates their function. Ali *et al.*[87] reported that the binding of chemokines to peptidoglycans is not a prerequisite for their function, but might enhance their bioactivity. MIP-1α and MIP-1β elicit local inflammatory response *in vitro*. MIP-1α and MIP-1β show chemotactic activities on lymphocytes using a microchemotaxis system.[88] In the central nervous system, where leukocytes exhibit chemokine receptors, MIP-1α and MCP-1 show high affinity to astrocytes after stimulation with IL-1β and TNF-α.[89] This suggests a role of these chemokines in central nervous system inflammation. To investigate *in vivo* activities of human MIP-1α, Lee *et al.*[90] studied cell activation after intradermal injection of human subjects. Although no acute cutaneous or systemic reactions were observed, the activation of endothelial cells was suggested by the expression of E-selectin. During the tissue repair process, murine MIP-1α shows effective chemotactic activity on macrophages. So far, two chemokine receptors (CCR1 and CCR5) have been identified for one type of MIP-1α (LD78α), whereas three receptors were reported for another type

of MIP-1α (LD78β).[64] CCR5 is the only chemokine receptor that binds with MIP-1β;[64] thus, MIP-1α is a more potent attractant of mononuclear cells than MIP-1β.

The I-309 subgroup of the CC chemokines consisting of I-309, HCC-2, CKβ8, and SLC each contain at least six cysteines.[91-94] In I-309, HCC-2, and MPIF1, an additional cysteine is located within the sheet domain and a second one is located at the C-terminal helix. These cysteines may form a third disulfide bond anchoring the C-terminal helix to the sheet. SLC has two cysteines on the C-terminal domain. Eotaxin 2 has an unpaired fifth cysteine at the C-terminus.[92]

Chemokines in Fish: Fish chemokines can be grouped into three subfamilies; CC, CXC and C chemokines. The CX_3C chemokine subfamily has not been reported until now. The first fish chemokine, CK-1, was reported by Dixon *et al.*[95] from trout. CK-1 has four highly conserved cysteines of CC chemokine structure, with a gene structure consisting of 4 exons and 3 introns, which is more similar to CXC chemokines than to the CC chemokines. The Japanese flounder CC chemokine gene, in contrast, consists of 3 exons and 2 introns.[96] Kattiya *et al.*[97] identified a novel CC chemokine gene designated as *Paol*-SCYA104 in Japanese flounder and analysed its function. Also, trout CK-1 has two additional cysteines which are found in the C6-β chemokine subfamily.[75] Other fish CC chemokines have also been described based on their cDNA sequences. Fujiki *et al.* [98] reported one cDNA nucleotide sequence from carp leukocytes using suppression subtractive hybridization (SSH). This cDNA showed more similarity to the mammalian monocyte chemotactic protein (MCP) than to the trout CK-1, including the tyrosine and aspartate residues which are conserved in human MCP-1. Suppression subtractive hybridization was also performed by Liu *et al.*[99] for cloning a novel CC chemokine gene

in rainbow trout (trout CK-2). Trout CK-2 showed four conserved cysteines resembling that of mammalian CC chemokines with 20% identity at the amino acid level. The amino acid sequence of trout CK-2 is 44% identical to carp CC chemokine,[100] which is higher than trout CK-1 identity (20%). In a recent report, Kuroda *et al.*[101] described several CC chemokines: seven types from cichlid fish and one type from cat shark. The detection of a cat shark CC chemokine implied the emergence of a CC chemokine even in cartilaginous fishes. Phylogenetic analysis of currently available chemokine sequences, however, does not reveal any clear evidence on the orthology of fish and human chemokines. Alignment of fish and human CC chemokines showed that only four cysteine residues are shared by all of its members. However, several residues are partially conserved among the majority of those CC chemokines, including a tyrosine, which plays a role in receptor binding in human MCP-1.[102] It should be noted that within human CC chemokines, human MCP-1 is the closest CC chemokine to fish CC chemokines, including Japanese flounder CC chemokine,[96] carp CC chemokine[99] and cichlid CC chemokines.[101]

CXC chemokines in fish were also described in terms of their sequences. The first CXC chemokine in fish, an IL-8 homolog in lamprey, was reported by Najakshin et al.[103] This CXC chemokine was screened using subtractive hybridization similar to carp CC chemokine and trout CK-2. Its amino acid sequence shares 32% similarity with human IL-8. This finding indicates that the CXC chemokine gene arose at the time of the appearance of jawless fish. Other fish CXC chemokines have also reported similarity with IL-8; e.g. Japanese flounder,[90] rainbow trout,[98,104-105] banded dogfish,[106] silver chimaera,[107] and carp.[108-109] In mammalian and chicken IL-8, there is the ELR motif

(Glu-Leu-Arg) immediately before the CXC sequence. The presence of an ELR motif is important for neutrophil attraction, as this affects its binding to specific receptors.[78] All fish CXC chemokines lack the ELR motif immediately upstream of the CXC sequence, this being replaced with other motifs, e.g. the DLR motif in trout IL-8.[105] The only reported C chemokine in fish was found in the pufferfish.[110] This C chemokine, designated *f*CL1, possesses both the third and fourth conserved cysteines similar to other chemokines, but has only one N-terminal cysteine. It is functional and is expressed in the eye, gill, heart, muscle, skin and stomach. It shows 28% identity to both the human chemokine CCL28 and the zebrafish chemokine CCL1, but does not show a human ortholog by the BLAST program. The authors suggested the possibility that the BLAST parameters may not be optimal for detecting the human ortholog, since it has a short encoded protein and there is divergence in distinct species of cytokine genes.

Fujiki *et al.*[98] reported several copies (at least four) of rainbow trout IL-8. In contrast, trout CK-1[95] and CK-2 [99] showed only one or two partial copies. Functional aspects of CXC chemokine action in fish have been demonstrated using human recombinant IL-8 in carp neutrophilic granulocyte-enriched leukocytes displaying chemotactic activity.[109]

1.3. CYTOKINES AND CHEMOKINES AS ADJUVANTS IN DNA IMMUNIZATIONS: Genetic immunization involves the direct *in vivo* transfection of antigen-encoding plasmid DNA into mammalian cells. The expression vector causes *de novo* production of antigens in transfected cells which, in turn, initiate an immune response. The use of plasmid DNA as vaccines or immunotherapeutics has been investigated in a number of species using a variety of infectious agents.[111] Plasmid DNA

vaccines were capable of eliciting humoral and cellular immune responses against the vaccine encoded antigen.[112] Plasmid DNA vaccines provide several advantages over other types of vaccine technologies currently in use: a) DNA vaccines, in contrast to conventional subunit vaccines, allow for the encoded antigen to be efficiently processed and presented by the major histocompatibility complex (MHC) class I antigen processing pathway, which is critical for the induction of $CD8^+$ T-cell mediated responses. In this way, plasmid DNA vaccines perform similarly to live viral vaccine vectors but without the associated safety concerns; b) Plasmid DNA vaccines are easily manipulated using standard molecular biology techniques and scalable manufacturing methods are readily available; c) Due to the relative simplicity and inherent stability of DNA vaccines it is likely that vaccine distribution and administration will prove to be cost effective and relatively easy; and d) DNA vaccines are inherently safe since most DNA vaccine plasmids exist in a circular form, they do not integrate into the host genome and fail to replicate.[112]

DNA vaccines can be delivered by a number of routes (i.e., intradermal, mucosal and intramuscular) and by variety of techniques, (i.e., via needle and syringe, gene gun). To date, the most commonly used delivery route has been via intramuscular inoculation. Following delivery of plasmid DNA into the muscle, the vaccine DNA is taken up primarily by monocytes and to some extent by bone marrow derived antigen presenting cells (APCs). Once taken up by APCs, the DNA encoded vaccine antigen is expressed and subsequently processed and presented to the immune system in association with MHC class I molecules. Foreign antigen expression and presentation by MHC class I molecules is necessary to prime naïve $CD8^+$ T-cells to become effector cells. In addition,

monocytes can secrete a foreign antigen for phagocytosis by professional APCs for presentation to the immune system in association with MHC class II molecules. Exogenous vaccine antigen expression thus results in the induction of antibody and $CD4^+$ T-helper cell immune responses[112]. A variety of approaches have been taken to augment and modulate immunogenicity and efficacy of plasmid DNA vaccines. These approaches have included targeting DNA vaccine plasmids to APCs, enhancing foreign antigen transcription and translation, targeting the expressed foreign antigen to APCs, enhancing foreign antigen processing and MHC-restricted presentation through ubiquitination. However the approach that seems to offer the most promise is the co-delivery of plasmid based genetic adjuvants, including cytokines, chemokines and co-stimulatory molecules. In 1993 it was demonstrated that the direct intramuscular injection of a cytokine expressing plasmid DNA resulted in the *in vivo* production of biologically active cytokine.[113]

Effect of cytokines targeting APCs: Vector-encoded antigens are most likely presented by professional APCs, such as dendritic cells, that are present throughout most mammalian tissues. Muscle cells express low levels of MHC class I determinants, but lack MHC class II molecules, such as B7.1 or B7.2, that are crucial for receptor binding to $CD4^+$ T cells and are required as second signals for T cell activation.[114] APCs, in contrast, express both MHC class I and class II determinants, as well as co-stimulatory molecules. Furthermore, APCs participate in inflammatory reactions by expressing cytokine receptors and secreting cytokines. These characteristics allow APC to become activated upon a number of signals that contribute to the initiation of an innate host response. Upon activation, APC up-regulate expression of both MHC class I and classII

determinants and co-stimulatory molecules, which facilitates the display of antigenic peptide-MHC complexes in a form recognizable by T cells. Upon activation, APCs also become mobile and migrate to draining lymphatic tissues, where they initiate T cell activation and production of cytokines including, IL-12 and IFNγ. The result is an activation of inflammatory cells, including macrophages, stimulation of specific lymphocytes such as B-cells and production of other cytokines, such as IFNα, IL-1, IL-6, TNF α and GM-CSF.[111] Granulocyte/macrophage-CSF, a cytokine secreted by multiple cells is able to stimulate and recruit professional APCs, which makes it exceedingly useful as an adjuvant to vaccines.[115]

Effect of cytokines on Th cells: Th cell response to genetic immunization by using cytokines that directly affect T cell activation was well studied in mammalian models. Specifically, coinjection of IL-2, IL-12, IL-4 and IL-10 , and GM-CSF along with a DNA vaccine construct encoding for simian immunodeficiency virus (SIV) gag/pol proteins resulted in increased levels of antigen specific-antibodies.[116]

Pro-inflammatory cytokines such as IL-1β, IL-8 and MIP-1α as adjuvants: IL-1 is a cytokine with very potent inflammatory and pyrogenic activity and as a result the clinical development of IL-1 as a safe and efficacious vaccine adjuvant may be difficult. Despite these concerns, the use of IL-1 as a DNA vaccine adjuvant has met with some success.[112] The ability of IL-1β to augment the immune response to a DNA vaccine expressed antigen was first explored by Hakim *et al.*[117] The plasmid expressed TSA/IL-1β fusion protein was shown to elicit an increased antigen-specific IgG response and increased survival after lethal tumor challege.[117] In contrast, the co-administration of a plasmid expressing the full length IL-1β protein with a DNA vaccine expressing a soluble form of

the bovine herpes virus-1 gD protein failed to augment gD-specific serum IgG titres.[112] The ability of IL-8 to modulate the immune response elicited by DNA vaccination was first demonstrated in the mouse model of HSV infection.[118] In this study IL-8 co-administration with a DNA vaccine expressing the HSV gD protein resulted in a more Th-1 like immune response characterized by a slight increase in gD-specific IgG antibody titres and increased survival after intravaginal HSV challenge. MIP-1α has been shown to up-regulate IFN-γ production by $CD4^+$ T-cells[119] suggesting that MIP-1α may be useful as genetic adjuvant by enhancing Th-1 like cell-mediated immune responses. The first study to evaluate MIP-1α as a genetic adjuvant was done in mice. The intranasal or intramuscular inoculation of a DNA encoding MIP-1α with a plasmid DNA expressing HIV-1 env resulted in increased serum IgG and increased mucosal IgG and IgA antibody responses.[120] In addition MIP-1α co-administration resulted in an increased IgG2a and decreased IgG1 antibody isotype response, as well as an increased DTH and CTL response characteristic of a Th1-like immune response.[120] Sin *et al.*[118] showed that the intramuscular co-administration of MIP-1α with a DNA vaccine expressing HSV-2 gD resulted in only a slight increase in the gD-specific serum IgG antibody response. In contrast, the intramuscular injection of MIP-1α with HSV-2 gD resulted in a more Th2-like immune response with increased IgG1, decreased IgG2a and increased mortality after intravaginal challenge with HSV-2. Eo *et al.*[121] working in the mouse model also showed that the intramuscular administration of MIP-1α with a DNA vaccine encoding HSV gB resulted in a more Th-1 like immune response and increased protection after intravaginal challenge with HSV-1 and also the route of cytokine administration may impact on the overall immunomodulatory effect of MIP-1α.

1.3.1. DNA vaccine and adjuvants technology in Aquaculture: Infectious diseases have been a major impediment to the development and profitability of fish farms. Vaccines offer the most efficient way to control infectious pathogens, but in the aquaculture industry they are in a relatively early phase of development. Some DNA vaccines have been developed for viral diseases such as Viral haemorrhagic septicaemia virus (VHSV) in Japanese flounder[122], Hirame rhabdovirus (HRV) in Japanese flounder,[123] Noda virus in turobot[124] and infectious haematopoietic necrosis virus (IHNV) in rainbow trout.[125] Adjuvants in DNA vaccinations in aquaculture have not been tried so far except for IL-1β.[126] Byon *et al.*[122] used the VHS glycoprotein gene (G) and showed that it was able to induce protective immunity against VHS that lasted for 21 days. The microarray analysis showed that the G gene induced non-specific immune response genes such as NK, Kupffer cell receptor, MIP-1α and MX1 protein gene and specific immune-related gene such as CD 20 receptor, CD8 alpha chain, CD40 and B lymphocyte cell adhesion molecule. Takano *et al.*[123] developed a DNA vaccine using HRV glycoprotein gene (HRV g) which provided strong protection against hirame rhabdovirus in Japanese flounder. They also conducted a real- time PCR analysis to quantify immune related genes such as MHC class Iα, IIα, IIβ, TCR-α, β1, β2 and γ to characterize the immune response at 1 and 7 days after DNA vaccination and found that the copy numbers were at least 2-fold higher than those of non-vaccinated fish. Sommerset *et al.*[124] developed a DNA vaccine for viral nervous necrosis in turbot using VHSV glycoprotein gene. They demonstrated the cross protection ability of the rhabdoviral glycoprotein genes against noda virus challenge in turbot. Rainbow trout vaccinated with DNA expressing the G protein of infectious haematopoietic necrosis virus had a relative percentage survival

(RPS) of 75% with live virus challenge.[125] Yin and Kwang[126] demonstrated the immuno adjuvant properties of IL-1β in carp against *Aeromonas hydrophila* and found that the mean agglutinating antibody titre at 3 weeks post-vaccination showed that that the titre of the titre of the immune group injected with killed *A.hydrophila* cells plus recombinant IL-1β peptide was significantly higher than that of the group injected with killed bacterial cells alone.

1.4. OBJECTIVES OF THE PRESENT STUDY

The direct injection of a naked plasmid DNA vaccine encoding a foreign antigen results in plasmid uptake and protein expression leading to the induction of antigen-specific cellular and humoral immune responses. One approach to improve the immunogenicity of DNA vaccines is through the co-delivery of cytokine expression plasmids as adjuvants. Plasmid DNA-encoded immunomodulatory cytokines not only alter the magnitude and direction of the DNA vaccine-elicited immune response, but can also improve vaccine efficacy. In the present study, we used IL-1β, IL-8 and JFCC1 as genetic adjuvants in viral haemorrahagic septicemia virus glycoprotein (VHSg) DNA vaccine to enhance the efficiency of glycoprotein gene. The second part of the strategy was to estimate the immunomodulatory properties and the expression pattern of various immune-related genes induced in response to these three cytokines by using cDNA microarrays.

1.5. REFERENCES

1. Vannuccini S. Overview of fish production, utilization, consumption and trade. *FAO bulletin* 2003
2. FAO fisheries department. World review of fisheries and aquaculture. In: The state of world fisheries and aquaculture. Rome: FAO, 2002. P. 3-52
3. Noble AC, Summerfelt ST. Diseases encountered in rainbow trout cultured in re-circulating systems. *Annu. Rev. Fish dis.* 1996; **6:** 65-92
4. Balebona MC, Zorilla I, Morinigo MA, Borrego. Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata L.*) in south western Spain from 1991-1996. *Aquaculture* 1998; **166:** 19-35
5. Zarkadis IK, Mastellos D, Lambris JD. Phylogenetic aspects of the complement system. *Dev. Comp. Immunol.* 2001; **25:** 745-762
6. Abbas AK, Lichtman AH, Pober JS. Cellular and Molecular Immunology, 4th Edition. 2000 p 4-5
7. Jones SRM. The occurrence and mechanisms of innate immunity against parasites in fish. *Dev. Comp. Immunol.* 2001; **25:** 841-852
8. Bols NC, Brubacher JL, Ganassin RC, Lee LEJ. Ecotoxicology and innate immunity in fish. *Dev. Comp. Immunol.* 2001; **25:** 853-873
9. Takahashi H. Antigen presentation in vaccine development. *Comp. Immunol. Microbiol. Infect. Dis.* 2003; **26:** 309-328
10. Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. *Trends Immunol.* 2002; **23:** 201-208
11. Zubler RH, Lowenthal JW, Erard F, Hashimoto N, Devos R, MacDonald HR.

Activated B cells express receptors for, and proliferate in response to, pure interleukin 2. *J. Exp. Med.* 1984; **160:** 1170-1183

12. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naïve, central memory and effector memory $CD4^+$ T cells. *Pathol. Biol.* 2003; **51:** 64-66
13. Sung JL, Lin JT, Gorham JD. CD28 co-stimulation regulates the effect of transforming growth factor-β1 on the proliferation of naïve CD4+ T cells. *Int. Immunopharmacol.* 2003; **3:** 233-245
14. Sprent J, Surh CD. Cytokines and T cell homeostasis. *Immunol. Lett.* 2003; **85:** 145-149
15. Sprent J, Surh CD. Generation and maintenance of memory T cells. *Curr. Opin. Immunol.* 2001; **13:** 248-254
16. Grogan JL, Locksley RM. T helper cell differentiation: on again, off again. *Curr. Opin. Immunol.* 2002; **14:** 366-372
17. DeLuca D, Clark DR. Interleukin-7 negatively regulates the development of mature T cells in fetal thymus organ cultures. *Dev. Comp. Immunol.* 2002; **26:** 365-384
18. Mukamoto M, Kodama H, Baba T. Effects of cytokines from thymocytes and thymic stromal cells on chicken intrathymic T cell development. *Vet. Immunol. Immunopathol.* 1999; **67:** 223-233
19. Norment AM, Bevan MJ. Role of chemokines in thymocyte development. *Semin. Immunol.* 2000; **12:** 445-455
20. Liu CC, Perussia B, Young JDE. The emerging role of IL-15 in NK-cell development. *Immunology Today* 2000; **21:** 113-116

21. Müller G, Lipp M. Concerted action of the chemokine and lymphotoxin system in secondary lymphoid-organ development. *Curr. Opin. Immunol.* 2003; **15:** 217-224

22. Secombes CJ, Zou J, Laing K, Daniels DG, Cunningham C. Cytokine genes in fish. *Aquaculture* 1999; **172:** 93-102

23. Secombes CJ, Wang T, Hong S, Peddie S, Crampe M, Laing KJ, Cunningham C, Zou J. Cytokines and innate immunity of fish. *Dev. Comp. Immunol.* 2001; **25:** 713-723

24. Hirono I, Nam BH, Kurobe T, Aoki T. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus. J. Immunol.* 2000; **165:** 4423-4427

25. Laing KJ, Wang T, Zou J, Holland J, Hong S, Bols N, Hirono I, Aoki T, Secombes CJ. Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* tumor necrosis factor-α. *Eur. J. Biochem.* 2001; **268:** 1315-1322

26. Zou J, Secombes CJ, Long S, Miller N, Clem LW, Chinchar VG. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). *Dev. Comp. Immunol.* 2003; **27:** 845-858

27. Zou J, Wang T, Hirono I, Aoki T, Inagawa H, Honda T, Soma GI, Ototake M, Nakanishi T, Ellis AE, Secombes CJ. Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss. Dev.Comp. Immunol.* 2002; **26:** 161-172

28. Yin Z, Kwang J. Molecular cloning and characterization of carp transforming growth factor β1 from activated leucocytes. *Fish Shellfish Immunol.* 2000; **10:** 309-318

29. Tafalla C, Aranguren R, Secombes CJ, Castrillo JL, Novoa B, Figueras A. Molecular

characterisation of sea bream (*Sparus aurata*) transforming growth factor β1. *Fish Shellfish Immunol.* 2003; **14:** 405-421

30. Hughes AL. Evolution of the interleukin-1 gene family in mammals. *J.Mol.Evol.* 1994; **39:** 6-12
31. Dinarello CA. Interleukin-1. *Cytokine Growth Factor Rev.* 1997; **8:** 253-256
32. Secombes CJ, Bird S, Cunnigham C, Zou J. Interleukin-1 in fish. *Fish shellfish Immunol.* 1999; **9:** 335-343
33. Zou J, Grabowski PS, Cunningham C, Secombes CJ. Molecular cloning of interleukin-1β from rainbow trout Oncorhynchus mykiss reveals no evidence of an ICE cut site. *Cytokine* 1999; **11:** 552-560
34. Fujiki K, Shin DH, Nakao M, Yano T. Molecular cloning and expression analysis of carp (Cyprinus carpio) interleukin-1β high affinity immunoglobulin E Fc receptor γ subunit and serum amyloid A. *Fish Shell fish.immunol.* 2000; **10:** 229-242
35. Scapigliati G, Buonocore F, Bird S, Zou J, Pelegrin P, Falasca C, Prugnoli D, Secombes CJ. Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1β. *Fish Shellfish Immunol.* 2001; **11:** 711-726
36. Pelegrin P, Castillo JG, Mulero V, Meseguer J. Interleukin -1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines. *Cytokine* 2001; **16:** 67-72
37. Laliberte RE, Eggler J, Gabel CA. ATP treatment of human monocytes promotes caspase-1 maturation and externalization. *J.Biol.Chem.* 1999; **274:** 36994-36951
38. Black RA, Kronheim RS, Cantrell M, Deeley MC, March CJ, Prickett KS, Wignal J,

Conlon PJ, Cosmon D, Hopp TP, Mochizuki DY. Generation of biologically active interleukin-1β by proteolytic cleavage of the inactive precursor. *J.Biol.Chem.* 1988; **263:** 9437-9442

39. Dinarello CA. Biological basis for interleukin-1 in disease. *Blood* 1996; **87:** 2095-2147

40. Yuan J, Shaam S, Ledoux S, Ellis HM, Horvitz HR. The *C.elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell* 1993; **75:** 641

41. Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A. Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc.Natl.Acad.Sci.USA.* 1986; **83:** 1670-1674

42. Engelsma MY, Stet RJM, Schipper H, Verburg-van Kemenade BML. Regulation of interleukin-1 beta expression in the common carp, *Cyprinus carpio L. Dev. Comp. Immunol.* 2001; **25:** 195-203

43. Zou J, Cunningham C, Secombes CJ. The rainbow trout *Oncorhynchus mykiss* interleukin-1β gene has a different organization to mammals and undergoes incomplete splicing. *Eur.J.Biochem.* 1999; **259:** 901-908

44. Jarrous N, Kaempfer R. Induction of human interleukin-1 gene expression by retinoic acid and its regulation at processing of precursor transcripts. *J.Biol.Chem.* 1994; **269:** 23141-23149

45. Serkkola E, Hurme M. Synergism between protein kinase C and cAMP dependent pathways in the expression of the interleukin-1β gene is mediated via the activator protein-1 (AP-1) enhancer activity. *Eur.J.Biochem.* 1993; **213:** 243-249

46. Vannier E, Dinarello CA. Histamine enhances interleukin-1-induced IL-1 gene expression and protein synthesis via H2 receptors in peripheral blood mononuclear cells: comparison with IL-1 receptor antagonist. *J.Clin. Invetst.* 1993; **92:** 281-287

47. Schindler R, Clark BD, Dinarello CA. Dissociation between interleukin-1β mRNA and protein synthesis in human peripheral blood mononuclear cells. *J.Biol.Chem.* 1990; **265:** 10232-10237

48. Schindler R, Gelfand JA, Dinarello CA. Recombinant C5a stimulates transcription rather than translation of IL-1 and TNF; cytokine synthesis induced by LPS, IL-1 or PMA. *Blood* 1990; **76:** 1631-1638

49. Stevenson FT, Bursten SL, Fanton C, Locksley RM, Lovett DH. The 31-kDa precursor of interleukin 1α is myristoylated on specific lysines within the 16-kDa N-terminal propiece. *Proc.Natl.Acad.Sci.USA* 1993; **90:** 7245-7249

50. Jobling SA, Auron PE, Gurka G, Webb AC, McDonald B, Rosenwasser LJ, Gehrke L. Biological activity and receptor binding of human prointerleukin-1β and subpeptides. *J.Biol.Chem.* 1988; **263:** 16372-16378

51. Dinarello CA. Interleukin-1 and Interleukin-1 antagonism. *Blood* 1991; **77:** 1627-1652

52. Vannier E, Miller LC, Schindler R, Terlain B, Dinarello CA. Histamine suppresses the synthesis of tumour necrosis factor-α *in vitro* via H_2 receptors. *Cytokine* 1989; **1:** 123-129

53. Warner SJC, Auger KR, Libby P. Human interleukin-1 induces interleukin-1 gene expression in human vascular smooth muscle cells. *J. Exp.Med.* 1987; **165:** 1316-1331

54. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MA, O' Connor JV. Tumor necrosis factor (Cachectin) is an endogenous pyrogen and induces production of interleukin-1. 1986; **163:** 1433-1450

55. Hart PH, Vitty GF, Burgess DR, whitty GA, Piccoli DS, Hamilton JA. Potential anti-inflammatory effects of interleukin-4: suppression of human monocyte tumor necrosis factor, interleukin-1 and prostaglandin E_2. *Proc.Natl.Acad.Sci.USA.* 1989; **86:** 3803-3808

56. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1 and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood* 1990; **75:** 40-47

57. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NFκB activity through induction of IκB synthesis. *Science* 1995; **270:** 286-290

58. Marsden MJ, Vaughan LM, Foster TJ, Secombes CJ. A live (aro A) *Aeromonas salmonocida* vaccine for furunculosis preferentially stimulates T-cell responses relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*). *Inferction and Immunity* 1996; **64:** 3863-3869

59. Zou J, Holland J, Pleguezuelos O, Cunnigham C, Secombes CJ. Factors influencing the expression of interleukin-1β in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. *Dev.Comp.Immunol.* 2000; 24: 5 75-582

60. Tucker C, Endo M, Hirono I, Aoki T. Assessment of DNA vaccine potential for juvenile Japanese flounder, *paralichthys olivaceus*, through the introduction of

reporter genes by particle bombardment and histopathology. *Vaccine* 2001; **19**: 801-809

61. Kono T, Fujiki K, Nakao M, Yano T, Endo M, Sakai M. The immune responses of common carp, *Cyprinus carpio L.*, injected with carp interlekin-1β gene. *J. Interferon Cytokine Res.* 2002; **22**: 413-419
62. Ward SG, Westwick J. Chemokines: understanding their role in T-lymphocyte biology. *Biochem. J.* 1998; **333:** 457-470
63. Moser B, Ebert L. Lymphocyte traffic control by chemokines: follicular B helper T cells. *Immunol. Lett.* 2003; **85:** 105-112
64. Rollins BJ. Chemokines. *Blood* 1997; **90:** 909-928
65. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR. Zlotnik A, Schall TJ. A new class of membrane-bound chemokine with the CX_3C motif. *Nature* 1997; **385:** 640-644
66. Kelner GS, Kennedy J, Bacon KB, Kleyensteuber S, Largaespada DA, Jenkins NA, Copel, NG, Bazan JF, Moore KW, Schall TJ. Lymphotactin: a cytokine that represents a new class of chemokine. *Science* 1994; **266:** 1395-1399
67. Hughes AL, Yeager M. Coevolution of the mammalian chemokines and their receptors. *Immunogenetics* 1999; **49:** 115-124.
68. Goh CS, Bogan AA, Joachimiak M, Walther D, Cohen FE. Co-evolution of proteins with their interaction partners. *J. Mol. Biol.* 2000; **299:** 283-293
69. Sick C, Schneider K, Staeheli P, Weining KC. Novel chicken CXC and CC chemokines. *Cytokine* 2000; **12:** 181-186
70. Hughes S, Haynes A, O'Regan M, Bumstead N. Identification, mapping, and

phylogenetic analysis of three novel chicken CC chemokines. *Immunogenetics* 2001; **53:** 674-683

71. Lam KM. The macrophage inflammatory protein-1β in the supernatants of *Mycoplasma gallisepticum*-infected chicken leukocytes attracts the migration of chicken heterophils and lymphocytes. *Dev. Comp. Immunol.* 2002; **26:** 85-93
72. Braun M, Wunderlin M, Spieth K, Knochel W, Gierschik P, Moepps B. *Xenopus laevis* Stromal cell-derived factor 1: conservation of structure and function during vertebrate development. *J. Immunol.* 2002; **168:** 2340-2347
73. Proudfoot AEI, Power CA, Hoogewerf AJ, Montjovent MO, Borlat F, Offord RE, Wells TNC. Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J. Biol. Chem.* 1996; **271:** 2599-2603
74. Gong JH, Clark-Lewis I. Antagonists of monocyte chemoattractant protein 1 identified by modification of functionally critical NH2-terminal residues. *J. Exp. Med.* 1995; **181:** 631-640
75. Baggiolini M. Chemokines and leucocyte traffic. *Nature* 1998; **392:** 565-568
76. Koopmann W, Ediriwickrema C, Krangel MS. Structure and function of the glycosaminoglycan binding site of chemokine macrophage-inflammatory protein-1β. *J. Immunol.* 1999; **63:** 2120-2127
77. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, Sahagan BG, Neote K. Interferon-inducible T cell α chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J. Exp. Med.*

1998; **187:** 2009-2021

78. Clark-Lewis I, Schumacher C, Baggiolini M, Moser B. Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. *J. Biol. Chem.* 1991; **266:** 23128-23134

79. Moser B, Dewald B, Barella L, Schumacher C, Baggiolini M, Clark-Lewis I. Interleukin-8 Antagonists generated by N-terminal modification. *J. Biol. Chem.* 1993; **268:** 7125-7128

80. Jones SA, Dewald B, Clark-Lewis I, Baggiolini M. Chemokine antagonists that discriminate between interleukin-8 receptors. Selective blockers of CXCR2. *J. Biol. Chem.* 1997; **272:** 16166-161669

81. Loppnow H, Bil R, Hirt S, Schonbeck U, Herzberg M, Werdan K, Rietschel ET, Brandt E, Flad HD. Platelet-derived interleukin-1 induces cytokine production, but not proliferation of human vascular smooth muscle cells. *Blood* 1998; **91:** 134-141

82. Nagasawa T, Nakajima T, Tachibana K, Iizasa H, Bleul CC, Yoshie O, Matsushima K, Yoshida N, Springer TA, Kishimoto T. Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry co-receptor fusion. *Proc. Natl. Acad. Sci. U.S.A.* 1996; **93:** 14726-14729

83. Berkhout TA, Sarau HM, Moores K, White JR, Elshourbagy N, Appelbaum E, Reape RJ, Brawner M, Makwana J, Foley JJ, Schmidt DB, Imburgia C, McNulty D, Matthews J, O'Donnell K, O'Shannessy D, Scott M, Groot PH, Macphee C. Cloning,

in vitro expression, and functional characterization of a novel human CC chemokine of the monocyte chemotactic protein (MCP) family (MCP-4) that binds and signals through the CC chemokine receptor 2B. *J. Biol. Chem.* 1997; **272:** 16404-16413

84. Ben-Baruch A, Michiel DF, Oppenheim JJ. Signals and receptors involved in recruitment of inflammatory cells. *J. Biol. Chem.* 1995; **270:** 11703-11706

85. Menten P, Wuyts A, Van Damme J. Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 2002; **13:** 455-481

86. Irving SG, Zipfel PF, Balke J, McBride OW, Morton CC, Burd PR, Siebenlist U, Kelly K. Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q. *Nucleic Acids Res.* 1990; **18:** 3261-3270

87. Ali S, Palmer AC, Banerjee B, Fritchley SJ, Kirby JA. Examination of the function of RANTES, MIP-1α, and MIP-1β following interaction with heparin-like glycosaminoglycans. *J. Biol. Chem.* 2000; **275:** 11721-11727

88. Schall TJ, Bacon K, Camp RD, Kaspari JW, Goeddel DV. Human macrophage inflammatory protein alpha (MIP-1α) and MIP-1β chemokines attract distinct populations of lymphocytes. *J. Exp. Med.* 1993; **177:** 1821-1826

89. Andjelkovic AV, Kerkovich D, Shanley J, Pulliam L, Pachter JS. Expression of binding sites for β-chemokines on human astrocytes. *Glia* 1999; **28:** 225-235

90. Lee EY, Park HH, Kim YT, Choi TJ. Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralichthys olivaceous*). *Gene* 2001; **274:** 237-243

91. Miller MD, Krangel MS. The human cytokine I-309 is amino cytochemoattractant.

Proc.Natl. Acad. Sci. U.S.A. 1992; **89:** 2950-2954

92. Nagira M, Imai T, Hieshima K, Kusuda J, Ridanpaa M, Takagi S, Nishimura M, Kakizaki M, Nomiyama H, Yoshie O. Molecular cloning of a novel human CC chemokine secondary lymphoid-tissue chemokine that is a potent chemoattractant for lymphocytes and mapped to chromosome 9p13. *J. Biol. Chem.* 1997; **272:** 19518-19524
93. Pardigol A, Forssmann U, Zucht HD, Loetscher P, Schulz-Knappe P, Baggiolini M, Forssmann WG, Magert HJ. HCC-2, a human chemokine: gene structure, expression pattern, and biological activity. *Proc. Natl. Acad. Sci. U.S.A.* 1998; **95:** 6308-6313
94. Youn BS, Zhang SM, Broxmeyer HE, Cooper S, Antol K, Fraser M. Jr, Kwon BS. Characterization of CKβ8 and CKβ8-1: two alternatively spliced forms of human β-chemokine, chemoattractants for neutrophils, monocytes, and lymphocytes, and potent agonists at CC chemokine receptor 1. *Blood* 1998; **91:** 3118-3126
95. Dixon B, Shum B, Adams AJ, Magor KE, Hedrick RO, Muir DG, Parham P. CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). *Immunol. Rev.* 1998; **166:** 341-348
96. Kono T, Kusuda R, Kawahara E, Sakai M. The analysis of immune responses of a novel CC chemokine gene from Japanese flounder *Paralichthys olivaceus*. *Vaccine* 2003; **21:** 446-457
97. Khattiya R, Ohira T, Hirono I, Aoki T. Identification of a novel Japanese flounder (*Paralichthys olivaceus*) CC chemokine gene and analysis of its function. *Immunogenetics* 2004; **55:** 763-769

98. Fujiki K, Gauley J, Bols NC, Dixon B. Genomic cloning of novel isotypes of the rainbow trout interleukin-8. *Immunogenetics* 2003; **55:** 126-131

99. Liu L, Fujiki K, Dixon B, Sundick RS. Cloning of a novel rainbow trout (*Oncorhynchus mykiss*) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDNA fragments containing AU-rich elements. *Cytokine* 2002; **17:** 71-81

100. Fujiki K, Shin DH, Nakao M, Yano T. Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. *Immunogenetics* 1999; **49:** 909-914

101. Kuroda N, Uinuk-Ool TS, Sato A, Samonte IE, Figueroa F, Mayer WE, Klein J, Identification of chemokines and a chemokine receptor in cichlid fish, shark, and lamprey. *Immunogenetics* 2003; **54:** 884-895

102. Zhang YJ, Rutledge BJ, Rollins BJ. Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis. Identification of a mutated protein that inhibits MCP-1-mediated monocyte chemotaxis. *J. Biol. Chem.* 1994; **269:** 15918-15924

103. Najakshin AM, Mechetina LV, Alabyev BY, Taranin AV. Identification of an IL-8 homolog in lamprey (*Lampetra fluviatilis*): early evolutionary divergence of chemokines. *Eur. J. Immunol.* 1999; **29:** 375-382

104. Sangrador-Vegor A, Lennington JB, Smith TJ. Molecular cloning of an IL-8-like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression subtractive hybridization. *Cytokine* 2002; **17:** 66-70

105. Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T, Secombes CJ. Identification and analysis of an interleukin 8-like molecule in rainbowtrout, *Oncorhynchus mykis*. *Dev. Comp. Immunol.* 2002; **26:** 433-444

106. Inoue Y, Haruta C, Usui K, Moritomo T, Nakanishi T. Molecular cloning and sequencing of the banded dogfish (*Triakis scyllia*) interleukin-8 cDNA. *Fish Shellfish Immunol.* 2003a; **14:** 275-281

107. Inoue Y, Endo M, Haruta C, Taniuchi T, Moritomo T, Nakanishi T. Molecular cloning and sequencing of the silverchimera (*Chimaera phantasma*) interleukin-8 cDNA. *Fish Shellfish Immunol.* 2003b; **15:** 269-274

108. Savan R, Kono T, Aman A, Sakai M. Isolation and characterization of CXC chemokine in common carp (*Cyprinus carpio* L.). *Mol. Immunol.* 2003; **39:** 829-834

109. Huising MO, Stolte E, Flik G, Savelkoul HF, Verburg-van Kemenade BM. CXC chemokines and leukocyte chemotaxis in common carp (*Cyprinus carpio* L.). *Dev. Comp. Immunol.* 2003; **27:** 875-888

110. Gilligan P, Brenner S, Venkatesh B. Fugu and human sequence comparison identifies novel human genes and conserved non-coding sequences. *Gene* 2002; **294:** 35-44

111. Pasquini S, Xiang Z, Wang Y, He Z, Deng H, Blaszczyk-Thurin M, Ertl HCJ. Cytokines and costimulatory molecules as genetic adjuvants. Immunol. *Cell Biol.* 1997; **75:** 397-401

112. Egan MA, Israel ZR. The use of cytokines and chemokines as genetic adjuvants for plasmid DNA vaccines. *Clinical and Applied Immunol.Rev.* 2002; **2:** 255-287

113. Raz E, Watanabe A, Baird SM, Eisenberg RA, Parr TB, Lotz M, Kipps TJ, Carson DA. Systemic immunological effects of cytokine genes injected into skeletal muscle. *Proc. Natl. Acad. Sci. USA 1993;* **90:** 4523-4527

114. Nikcevich KM, Gordon KB, Tan L, Hurst SD, Kroepfl JF, Gardiner M, Barrett TA, Miller SD. IFN-gamma-activated primary murine astrocytes express B7 co-stimulatory molecules and prime naïve antigen-specific T cells. *J. Immunol.* 1997; 158: 614-621

115. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin-4 and downregulated by tumor necrosis factor α. *J. Exp.Med.* 1994; **179:** 1109-1118

116. Kim JJ, Simbiri KA, Sin JI, Dang K, Oh J, Dentchev T, Lee D, Nottingham KL, Chalian AA, McCallus D, Ciccarelli R, Agadjanyan MG, Weiner DB. Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J.Interferon Cytokine Res.* 1999; **19:** 77-84

117. Hakim I, Levy S, Levy R. A nine amino acid peptide from IL-1beta augments antitumor responses induced by protein and DNA vaccines. *J.Immunol.* 1996; **157:** 5503-5511

118. Sin J, Kim JJ, Arnold RL, Shroff KE, McCallus D, Pachuk C, Satishchandran C, Weiner DB. DNA vaccines encoding interleukin-8 and RANTES enhance antigen specific Th-1 type $CD4^+$ T-cell mediated protective immunity against herpes simplex virus type 2 *in vivo*. *J. virol.* 2000; **74:** 11173-11180

119. Karpus W, Lukacs N, Kennedy K, Smith W, Hurst S, Barrett T. Differential CC-

chemokine-induced enhancement of T helper cell cytokine production. *J.Immunol.* 1997; **158:** 4129-4136

120. Lu Y, Xin KQ, Hamajima K, Tsuji T, Aoki I, Yang J, Sasaki S, Fukushima J, Yoshimura T, Toda S, Okada E, Okuda K. Macrophage inflammatory protein-1alpha (MIP-1α) expression plasmid enhances DNA vaccine-induced immune response against HIV-1. *Clin. Exp. Immunol.* 1999; **115:** 335-341
121. Eo SK, Lee S, Chun S, Rouse BT. Modulation of immunity against herpes simplex virus infection via mucosal genetic transfer of plasmid DNA encoding chemokines. *J.Virol.* 2001; **75:** 569-578
122. Byon JY, Ohira T, Hirono I, Aoki T. Use of a cDNA microarray to study immunity against viral hermorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. *Fish shellfish Immunol.* 2005; **18:** 135-147
123. Takano T, Iwahori A, Hirono I, Aoki T. Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after vaccination. *Fish shellfish Immunol.* 2004; **17:** 367-374
124. Sommerset I, Lorenzen E, Lorenzen N, Bleie H, Nerland AH. A DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot. *Vaccine* 2003; **21:** 4661-4667
125. Anderson ED, Mourich DV, Fahrenkrug SC, Lapatra S, Shepherd J, Leong JC. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious haematopoitic necrosis virus. *Mol.Mar.Biol.Biotechnol.* 1996; **5:** 114-122
126. Yin Z, Kwang J. Carp interleukin-1β in the role of an immuno-adjuvant. *Fish shellfish Immunol.* 2000; **10:** 375-378

Chapter 2

Transcriptional profile of IL-1β induced kidney cells of Japanese flounder, *Paralichthys olivaceus*

ABSTRACT: IL-1β cDNA of Japanese flounder was found to consist of 1,329 bp, encoded 247 amino acid residues. Among the fish IL-1βs in the databases, the one with the highest identity of Japanese flounder IL-1β was that of seabass (62% identity). The expression of IL-1β was induced by treatment with ConA/PMA and LPS. The copy number of IL-1β mRNA was increased 30 fold after stimulation with ConA/PMA. Of 871 cDNAs on a microarray, 93 genes (10.7%) were up-regulated or down-regulated by IL-1β at 1, 3 and 7 days post injection. The induced gene expression was highest on 1 day followed by 3 day and 7 day. Seven percent of known and 3.7% of unknown genes of the 871 tested genes were differentially expressed. Of the genes tested, 7.4% were up-regulated and 3.3% were down regulated. Cytokine genes such as tumor necrosis factor, G-CSF and chemokine receptor A were induced in response to IL-1β. Cell surface antigens such as IgM, MHC class I and CD20 receptor were up-regulated. Signal transduction genes such as Toll-like receptor 1 and SH3P2 were also up-regulated. The glucocorticoid receptor and cAMP early repressor were down regulated in our microarray analysis.

KEY WORDS: **IL-1β, Japanese flounder, gene expression, microarray, real time PCR.**

2.1. INTRODUCTION

Interleukin-1β is an IL-1 cytokine with a beta trefoil structure that is composed of 12 beta sheets, [1] and plays a pivotal role in the inflammatory response as well as in the maturation and proliferation of many immune cell types.[2] Recently, IL-1β cDNAs and genes have been cloned from different fish species.[3] Expression of several humoral and cellular factors, production of reactive metabolites and secretion of hostile molecules have been suggested to depend on the expression of IL-1β in mammals.[4]

DNA microarray technology is a latest technology to determine the expression profile of many immune related genes of Japanese flounder in a short period of time. The most important application of microarrays is in the study of differential gene expression in disease and health, and in normal and abnormal physiological and immunological responses.[5] Microarrays thus identifying all the genes that are turned on at the site of infection *in vivo*. They can also be used to study the response of a host to challenge with the pathogen such as a cytokine or gene pathways, signaling pathways and identification of immune gene responses.[5] The microarray analysis would identify cytokine-responsive genes and help develop models for disease investigation.[6] A microarray analysis of IL-1β identified alterations in the expression of multiple transcription factors, cytokines, growth factors and their receptors, adhesion molecules, proteases and signaling intermediates that may contribute to inflammation in arthritis.[7]

In this study, we determined a full length cDNA of Japanese flounder IL-1β. The expression pattern of IL-1β in PBLs following stimulation with either ConA/PMA or LPS was carried out by real time PCR. We investigated the *in vivo* effects of IL-1β on the transcriptional program of Japanese flounder kidney by using cDNA microarrays to

obtain additional insights concerning the effect of this cytokine gene on the immune mechanism of Japanese flounder.

2.2. MATERIALS AND METHODS

2.2.1. Construction of cDNA library, screening and data analysis

A cDNA library was constructed from the peripheral blood leucocytes (PBLs) collected from Japanese flounder. The PBLs were isolated by centrifugation at 400xg for 20 min with Percoll solution density gradient (1.072 g/mL). The PBLs were cultured at 25^0C in RPMI 1640 medium containing LPS (500 μg/mL). mRNA was isolated from PBLs incubated in LPS after 1, 3, and 6 h, using a micro mRNA purification kit (Amersham Biotech, U.S.A) following the manufacturer's instructions. The purified mRNAs of three different time periods were pooled and used to construct a cDNA library. The cDNA library was synthesized using the SUPERSCRIPT^{TM} plasmid system (Life Technologies, U.S.A) following the instructions of the manufacaturer for cDNA synthesis and plasmid cloning.

A partial cDNA clone of IL-1β was used as a DNA probe to screen a full length cDNA of IL-1β. The hybridization was conducted as previously reported.[8] The determined nucleotide and deduced amino acid sequences, and multiple sequence alignments were analyzed by GENETYX ver.8.0 (SDC Software Development, Japan). Phylogeny was inferred using the Clustal X and PHYLIP program, and by distance analysis using the neighbor joining method.[9] The phylogenetic tree was generated using the Tree view software. The values supporting each node are derived from 100 re-samplings.

2.2.2. Construction of plasmid expressing JF- IL-1β

pGEM T-Easy vector (Promega, Madison, WI, USA) ligated with the full length Japanese flounder interleukin-1β(JF-IL-1β) cDNA, was sub cloned into pCI-neo mammalian expression vector (Promega, Madison, WI, USA) following the manufacturer's instructions. The plasmid was extracted by ultracentrifugation using a CsCl-ethidium bromide gradient.[10]

2.2.3. In vitro transcription / translation of JF-IL-1 β

The JF-IL-1 β cDNA was analyzed in a TNT quick coupled transcription/translation system (Promega, Madison, WI, USA). One microgram of the plasmid DNA was added to the kit components following the manufacturer's protocol. The reaction mixture was incubated at 30°C for 90 minutes and mixed with an equal volume of sodium dodecyl sulphate (SDS) buffer (125mM Tris hydrochloride,pH6.8, 2%SDS, 10%glycerol and 5% 2-mercaptoethanol) and boiled for 5 minutes. A 10μl portion of the mixture was run on a SDS-15%polyacryladamide gel with a 5%stacking gel. The gel was electro blotted to a polyvinylidene difluoride (PVDF) membrane (Atto, Tokyo, Japan), bound with streptavidine-alkaline phosphatase solution and calorimetrically detected with western blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI, USA).

2.2.4. Quantification of IL-1β by real- time PCR

The absolute copy number of the target transcript and standard curve for Japanese flounder IL-1β and β-actin were generated as described previously.[11] Briefly, a cloned plasmid DNA for each sample was used to generate a standard curve. The cloned plasmid DNA (0.5μL) was used in 50μL of PCR mixture. The PCR primers used in real-time PCR

are listed in Table. 1. The PCR-reacted products were purified using Amicon Microcon-PCR centrifugal filter devices (Millipore, USA).The copy number of reacted products were calculated according to the molecular weight of the products and then converted into the copy numbers based upon Avagadro's number (1 mol = 6.022 x 10^{23} molecules). Fifty microlitres of the PCR reaction was prepared for quantitative real-time PCR. The reaction mixture consisted of 5 μL template DNA (10 μg/mL), 5 μL of both forward and reverse primers (5 μm), 5 μL 10 x SYBR PCR buffer, 6 μL 25 mM $MgCl_2$, 4 μL dNTP blend (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dUTP), 0.25 μL AmpliTaq Gold (5 U/μL), 0.5 μL AmpErase UNG (1 U/μL) and 19.25 μL distilled water. The PCR amplification was performed as follows: 1 cycle at 50^0 C for 2 min and 95^0 C for 10 min. followed by 40 cycles at 95^0C for 15 s and 58^0C for 60 s. Thermal cycling and fluorescence detection was conducted using the Gene Amp 5700 sequence detection system as described above. All samples were run in triplicate with β-actin as an internal positive control[12] and the normalization reference for individual variation. Statistical analysis of expression levels between different samples was analyzed by an independent student's t-test. Values were considered to be significant when $P< 0.05$.

Table 1 Oligonucleotide primers used for real time PCR analysis

Name	Sequence(5'-3')	Information
IL-1Fst (Forward)	5'-cctgctcaacatcatgatga-3'	Designed for making standard curve in
IL-1Rst (Reverse)	5'-aagaactttgcggttgctgc-3'	real time PCR quantitative gene expression
β-actin Fst (Forward)	5'-tttccctccattgttggtcg-3'	analysis of IL-1β and β-actin
β-actin Rst (Reverse)	5'-gcgactctcagctcgttgta-3'	
IL-1β Frt (Forward)	5'-cgtctccaccagatcagttcag-3'	Designed for real time PCR analysis of
IL-1β Rrt (Reverse)	5'-gctgttctggaccagaatgagt-3'	IL-1β and β-actin.
β-actin Fst (Forward)	5'-tgatgaagcccagagcaga-3'	
β-actin Rst (Reverse)	5'-ctccatgtcatcccagttggt-3'	

2.2.5. Japanese flounder cDNA microarray construction

An 871 unique element Japanese flounder cDNA microarray was constructed based on the procedures described previously.[13] Briefly, the preparation of the DNA chip was performed as follows. Individual Japanese flounder cDNA clones were carefully chosen to avoid duplication of the same genes from our previous EST analyses. cDNA clones from ESTs were used as template DNA and the primers (sense- GTGCTGCAAGGCGATTAAGTTGG, antisense- TCCGGCTCGTATGTTGTGTGGA) were designed to anneal the vector region. The PCR was performed as follows. An initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 2 min, and a final elongation step at 72°C for 5 min. The resulting PCR products were purified and concentrated by 96-well PCR cleanup kit (Millipore, USA) to attain a final concentration above 500μg/mL. The purified PCR products were spotted on to the microarray slide by DNA Chip Research Inc, Tokyo, Japan. β-actin genes were included on the array as controls for labeling, hybridization and fluorescent background

2.2.6. Fish

Thirty six Japanese flounder fish, each weighing about 10 g were used in the study. The fish were maintained in recirculation sea water system at a constant temperature of 20°C. The fish were divided into two groups and intra-muscularly injected with 10μg of pCMV-JF-IL-1β or pCMV vector, respectively.

2.2.7. RNA preparation for microarray analysis

Total RNA was extracted from both pCMV-JF-IL-1β and pCMV vector kidney tissue of Japanese flounder at 1, 3, and 7 day post injection respectively, by TRIZOL reagent (Invitrogen Life technologies, USA) following the manufacturer's instructions.

mRNA was isolated from total RNA using Quick Prep micro mRNA purification kit (Amersham Biosciences, USA) according to the manufacturer's instructions.

2.2.8. Preparation of fluorescently labeled cDNA, and hybridization

One μg of pooled mRNA isolated from pCMV-IL-1β induced fish and pCMV vector injected fish were labeled with Cy5-amino-allyl dUTP and Cy3-amino-allyl dUTP, respectively, with a Labelstar array kit (Qiagen, USA) following the manufacturer's instructions. For final probe preparation, a sample containing equal amounts of Cy3- or Cy5-labeled cDNA was mixed with 7.5μL of hybridization buffer and 15 μL of formamide, transferred to the microarray glass slide, and incubated at 42°C for 18 h in a custom made slide chamber in which the humidity was maintained with few drops of distilled water. After hybridization, the arrays were washed with 2x SSC-0.1%SDS for 20 min at room temperature, then washed with 0.2x SSC-0.1% SDS for 20 min at room temperature, washed twice with 0.2x SSC-0.1% SDS for 20 min at 55^0C with gentle agitation, after rinsing with 0.2x SSC-0.1% SDS at room temperature, the slides were dried and scanned immediately using a GenePix 4000B scanner (Axon Instruments, Foster City, California, USA).

2.2.9. Signal detection and data analysis

The fluorescent intensity for each dye (Cy3/Cy5) was detected with a GenePix 4000B microarray scanner. Images were analyzed by GenePix pro 3.0 software provided with the scanner. The signal intensity was normalized to the signal intensity of β-actin by adjusting the PMT power and signal gain. Feature ratios of 2.0 and above was considered as up-regulation factor and 0.5 and below was considered as down regulation factor.

GenePix Pro 3.0 displays the data in tables that can be exported to any standard spread sheet program.

The nucleotide sequence reported for Japanese flounder interleukin-1β in the present paper has been submitted to the GenBank database and has been assigned the accession number as follows: GenBank accession no. AB070835.

2.2.10. Expression pattern of inducible genes in microarray analysis

Expression pattern of inducible genes was analyzed by cluster 3.0 software (Eisen, 2003).

2.3. RESULTS

2.3.1. Cloning and analysis of full length cDNA of JF-IL-1β

The Japanese flounder (JF) IL-1β cDNA consisted of 1,329 bp. The translated open reading frame gave a predicted 247 amino acid precursor peptide with a molecular weight of 28 kDa (Fig.1). The JF IL-1β cDNA has six complete TATTTA sequences and some other incomplete TATTTA sequences in the 3'UTR (GenBank acc.No.AB070835). The identities of the deduced amino acid sequence of Japanese flounder IL-1β to other species. IL-1βs ranging from 27% - 62% (Fig.2). Japanese flounder IL-1β has highest peptide similarity (62%) with seabass, followed by trout with 52% similarity. The amino acid

sequence alignment (Fig.2) showed a lack of aspartic acid residues in the region where ICE cuts the mammalian IL-1β precursor (Fig.2). Phylogenetic tree revealed that the Japanese flounder IL-1β branches with sea bass and trout as nearest neighbors, and mammals are more distant neighbors (Fig.3).

The molecular size of the Japanese flounder Interleukin-1 β (JF-IL-1 β) was analyzed by a coupled in vitro transcription-translation system. The presence of a major product of approximately 30 kD (Fig.4) corresponding to the pCMV-JF-IL-1 β confirmed that a full –size protein was expressed from the cDNA clone. This was in close agreement with the calculated molecular mass of the JF-IL-1 β at 28 kD.

2.3.2. Quantitative gene expression analysis following stimulation of leucocytes with ConA/PMA or LPS

As shown in Fig.5, all stimulated leukocytes were significantly induced as compared to normal leukocytes. The expression of IL-1β mRNA was significantly enhanced after stimulation with ConA/PMA for 1h and stimulation with LPS after 3h.

2.3.3. Up or down regulation of genes in cDNA microarray analyses

From the three arrays at 1, 3 and 7 day post injection, a total of 93 (10.7%) of the 871 tested genes were differentially expressed. Of the 871 tested genes, 61 known genes (7.0%) and 32 unknown genes (3.7%) were differentially regulated. A total of 64 genes or 7.4% were up-regulated and 29 genes or 3.3% were down regulated. The IL-1β - induced genes were grouped into 7 clusters based on their functional characteristics. Cytokines, such as tumor necrosis factor (TNF), Granulocyte colony stimulating factor (G-CSF) and Chemokine receptor A, are included in cluster 1. Cell surface antigens, such as MHC class I, IgM, CD20 are included in cluster 2. Cluster 3 is dominated by signal transduction genes, such as SH3P2, Toll like receptor. Apoptosis-related genes and inflammation-related genes are included in clusters 4 and cluster 5, respectively. Metabolism and other functionally regulated genes are included in cluster 6. The unknown/novel genes are grouped into cluster 7. Inducible cAMP early repressor and

glucocorticoid receptor genes were down regulated. The complete list of IL-1β-induced genes is presented in Table. 2.

Fig. 1

```
   1  gctgaatcgagaacactcacagaacctcctctccactgactaccacagatctttctccac  60
  61  tcagaaaaag atg gaatccaagatggaatgcaacgtgagccagatgtggagcgccaagat  120
                 M  E  S  K  M  E  C  N  V  S  Q  M  W  S  A  K  M
 121  gccacagggactgaacttggagatctcccatcacccgatgacaatgagaagtgtggtcaa  180
        P  Q  G  L  N  L  E  I  S  H  H  P  M  T  M  R  S  V  V  N
 181  cctcatcatcgccatggagcggctgaagggcagccattcggaatctgtgctgagcacaag  240
        L  I  I  A  M  E  R  L  K  G  S  H  S  E  S  V  L  S  T  S
 241  cttcacagatgaaaacctgctcaacatcatgatggagaacatcgtgnaagagcacattgt  300
        F  T  D  E  N  L  L  N  I  M  M  E  N  I  V  X  E  H  I  V
 301  gtgtgagagaagctcgtctccaccagatcagttcagcagaaggggcgtgtacacgtgcaa  360
        C  E  R  S  S  S  P  P  D  Q  F  S  R  R  G  V  Y  T  C  N
 361  catcactgacagccagaagaggaacttcattctggtccagaacagcatggagctccacgc  420
        I  T  D  S  Q  K  R  N  F  I  L  V  Q  N  S  M  E  L  H  A
 421  cgtgatgctgcagggaggcagcagcaaccgcaaagttcttctcaacatgtccacctatgt  480
        V  M  L  Q  G  G  S  S  N  R  K  V  L  L  N  M  S  T  Y  V
 481  gcacccttcacccaccatcgaagccaggcctgtcgttctgggcatcaaagacacagactt  540
        H  P  S  P  T  I  E  A  R  P  V  V  L  G  I  K  D  T  D  F
 541  cttcctgtcatgccagaagaatggtgcagagccaaccctgcatctggagcgtgtcgagaa  600
        F  L  S  C  Q  K  N  G  A  E  P  T  L  H  L  E  R  V  E  N
 601  caaatgcgacctggaggcattcagcagggacagtgagatggtgcgatttctgttctacaa  660
        K  C  D  L  E  A  F  S  R  D  S  E  M  V  R  F  L  F  Y  K
 661  gcaggacagcgggggggtgagcatcagcaccctcatgtcggcccgcttccccaactggta  720
        Q  D  S  G  G  V  S  I  S  T  L  M  S  A  R  F  P  N  W  Y
 721  catcagcacatcagagcaagacaacaggccagtgatggtgggtcagaagaatgcccggtg  780
        I  S  T  S  E  Q  D  N  R  P  V  M  V  G  Q  K  N  A  R  C
 781  ctaccagaccttcaacatccagcatcagagt taa agccggccagaggggaacgtggatca  840
        Y  Q  T  F  N  I  Q  H  Q  S  *
 841  gcatcttttcatttttgagcctcaatctgcagctattttaattcaaataatcagtcagga  900
 901  agatgtaatttcaaaagaagcatcaccactgtctgctggaaagactttgagtgtctagtt  960
 961  actgtagtaccgagtacagagagaaaaatgcttagtgcattgacatagtcaagttttaca  1020
1021  caaggtggcgttgttgagtagccagtgtatttcaaacagttactgctttaatc tatttat  1080
1081  a tatttaattata tatttatgtgtg tatttaaacatttgtgtttatttgggaatctgctt  1140
1141  actaaaaat tatttaatgatatgcagcaaa tatttaaaaaacctttcatattctgtgtt  1200
1201  gaatgtatgcttggagactgaaaaacaaaataagagtttcatcacatgctgtaaactgtg  1260
1261  tttatttgctgtgtttatgacaataaacatcttgaatcttgaaaaaaaaaaaaaaaaaaa  1320
1321  aaaaaaaaa  1329
```

Fig.2

```
Jflounder   1:----------------------------MESKMECNVSQMWSAKMPQGLNLEISHHPMTM 32
Seabass     1:----------------------------MESEMKCNMSEMWRSKMPQGLDLEITHHPLTM 32
Trout       1:----------------------MDFESNYSLIKNTSESAAWSSKLPQGLDLEVSHHPITM 38
Carp        1:MACHEYVHQLDLSEAFETDSAIYSDSADSDELDCPDPQSMSCQCDMHDIKLELSSHPHSM 60
Xenopus     1:-MALVPDLSSIPMEGYSGDDEMFYSDSPSGMKDDMEDAAQWQSSTSHCSLDIHVQITHGK 59
Chicken     1:-MAFVPDLD--VLESSSLSEETFYG--PSCLC--LQKKPRLDSE--HTTVDVQVTVRKGR 51
Human       1:-----MAEVPELASEMMAYYSGNEDDLFFEADGPKQMKCSFQDLDLCPLDGGIQLRISDH 55
                                                    . .     . ..   ..  .

Jflounder  33:RSVVNLIIAMERLKGSHSESVLSTSFTDENLLNIMMENIVXEHIVCERSSSPPD-----Q 87
Seabass    33:RRVVNLIIAMERLKGFSSETLMSTEFRDENLLNIMLESIVEEKIVFERGTTPTA-----Q 87
Trout      39:RHIANLI-AMERLKGGEGVT-MGTEFKDKDLLNFLLESAVEEHIVLELESAPPASRRAAG 96
Carp       61:RQVVNIIIAVERLKHIKNMS--SGKFCDEELLGFILENVIEERLVKPLNETPI------- 111
Xenopus    60:GSLHSFRKAVVLVVAVEKL-KR--GKERFFGDEDLLGLLDSIFVEEEIAFSQ-AKETHAS 115
Chicken    52:GA-RSFRRAAVLVVAMTKLLRR--PRSRDFADSDLSALLEEVF--EPVTFQR-LESSYAG 105
Human      56:HYSKGFRQAASVVVAMDKLRKMLVPCPQTFQENDLSTFFPFIFEEEPIFFDTWDNEAYVH 115
                 .   . . * ....           . .  ... ...   ..  . .    .

Jflounder  88:FSRRG-VYTCNITDSQKRNFI----LVQNSMELHAVMLQGGSSNRKVLLNMSTY---VHP 139
Seabass    88:YSKRR-EVQCSVTDSEKRSLV----LVPNSMELHAVMLQGGSDRCKVQLNMSTY---LDR 139
Trout      97:FSSTS-QYECSVTDSENKCWV----LMNEAMELHAMMLQGGSSYHKVHLNLSSY---VTP 148
Carp      112:YSKTSLTLQCTICDKYKKTMVQSNKLSDEPLHLKAVTLSAGAMQYKVQFSMSTF---VSS 168
Xenopus   116:ASTYRYQRATTCRIKDTSNKCFVMQKFHENAQLVALQLQGANIQREEKVSMAFY---ATQ 172
Chicken   106:APAFRYTRSQSFDIFDINQKCFVLES---PTQLVALHLQGPSSSQKVRLNIALYRPRGPR 162
Human     116:DAPVR---SLNCTLRDSQQKSLVMSG---PYELKALHLQGQDMEQQVVFSMSFV-----Q 164
                ^.  .    .  ..          .     .* *  *....   .. .... .

Jflounder 140:SPT-IEARPVVLGIKDTDFFLSC-QK-NGAE-PTLHLERVENKCDLEAFSRDSEMVRFLF 195
Seabass   140:TPS-AEAQTVALGIKGTNYYLSC-HK-DGEE-PTLHLEVVD-KASLANITSDSDMVRFLF 194
Trout     149:VPIETEARPVALGIKGSNLYLSC-SK-SGGR-PTLHLEEVADKDQLKSISQQSDMVRFLF 205
Carp      169:ATQ-KEAQPVCLGISNSNLYLAC-TQLDGSS-PVLILKEAS--GSVNTIKAGDPNDSLLF 223
Xenopus   173:PHQGGSKRPVALGLAGKNLYLSCRATEDGQDSPKLYLEEIS----NIKDVKGEDLNRFIF 228
Chicken   163:GSAGTGQMPVALGIKGYKLYMSC--VMSGTE-PTLQLEEAD----VMRDIDSVELTRFIF 215
Human     165:GEESNDKIPVALGLKEKNLYLSC--VLKDDK-PTLQLESVD----PKNYPKKKMEKRFVF 217
                    .. .*.**... .....*     .   *.* *...                ...*

Jflounder 196:YK--QDSGGVSISTLMSARFPNWYISTS--EQDNRPVMVGQKNAR-CYQTFNIQHQS--- 247
Seabass   195:YK--QDSG-LNISTLTSVPFSNWYISTA--EENNRPVQMCQESAR-RHRAFNIDNLKVDP 248
Trout     206:YR--RNTG-VDISTLESASFRNWFISTDMQQDYTKPVDMCQKAAPNRLTTFTIQRHN--- 259
Carp      224:FR--KETG-TRYNTFESVKYPGWFISTAFDDWEKVEMNQMPTTRTTNFTLEDQKRI---- 276
Xenopus   229:MKSQDGLNETSTNSFESVAFPGWYISTSQRENELVQMVHQKNQEAIKDFNLFSVI----- 283
Chicken   216:YR-LDSPTEGTTR-FESAAFPGWFICTSLQPRQPVGITNQPDQVNIATYKLSGR------ 267
Human     218:NK----IEINNKLEFESAQFPNWYISTSQAENMPVFLGGTKGGQDITDFTMQFVSS---- 269
               ..     .     ...*. ...*.*.*.  .   .

Jflounder 247:-------------                                               247
Seabass   249:TTEDQVCPLLNGQ                                               261
Trout     259:-------------                                               259
Carp      276:-------------                                               276
Xenopus   283:-------------                                               283
Chicken   267:-------------                                               267
Human     269:-------------                                               269
```

Fig.2 Alignment of Japanese flounder IL-1β with seabass, trout, carp, *xenopus*, chicken and human IL-1β. Sequences were obtained from DDBJ/EMBL/GenBank database.

Amino acids identical with Japanese flounder are shown by dots. The position of residues identical in all sequences are shown by asterisks. Gaps (dashes) have been placed to maximize identity. The ICE cut site (aspartic acid residue) in mammals is indicated with an arrow.

2.4. DISCUSSION

We determined the full length IL-1β cDNA sequence from a marine fish, Japanese flounder. Among previously reported fish IL-1β sequences in the databases, the one with the highest homology JF IL-1β is that of sea bass with 62% identity. JF IL-1β has multiple TATTTA motif sites in the 3' UTR, as is the case with other inflammatory molecules.[14] This suggests that JF IL-1β mRNA expression is tightly regulated. The presence of consensus sequence TATTTA in the 3'UTR of other fish IL-1βs [3-4] suggests that the regulation of mRNA stability is similar in other fish species. In mammals, IL-1β is produced as an inactive precursor that must be cleaved intracellularly by IL-1β converting enzyme (ICE).[15] In contrast to this fish IL-1β precursors cloned to date lack a clear ICE cut site [4], and this also appears to be the case for the analyzed Japanese flounder cDNA sequence.

Mitogen activation using ConA/PMA generates more lymphocyte clones than does antigenic LPS activation. This is because mitogens carry receptors with different antigen-binding specificities and therefore activate more clones. In this study, PMA was used in combination with ConA because ConA and PMA are not mitogenic when used alone.[16]

Fig.3 An unrooted phylogenetic tree showing the relationship between the full length Japanese flounder IL-1β amino acid sequence with other representative IL-1β sequences in different vertebrate groups. The tree was constructed by the neibour-joining method using the CLUSTAL X and PHYLIP packages, and was bootstrapped 100 times. The sequences were obtained from DDBJ/EMBL/GenBank database.

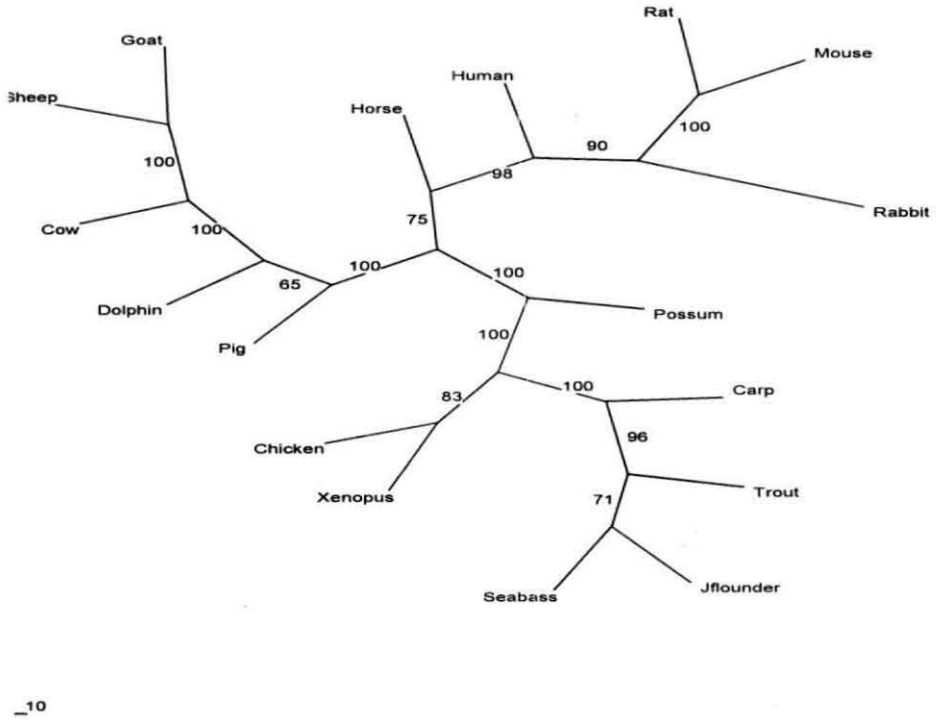


Fig.4 Translated protein of IL-1β.

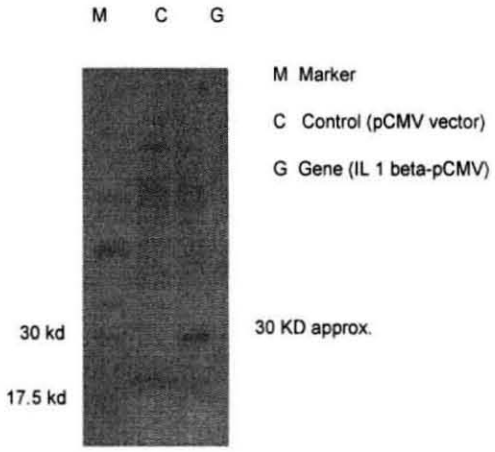
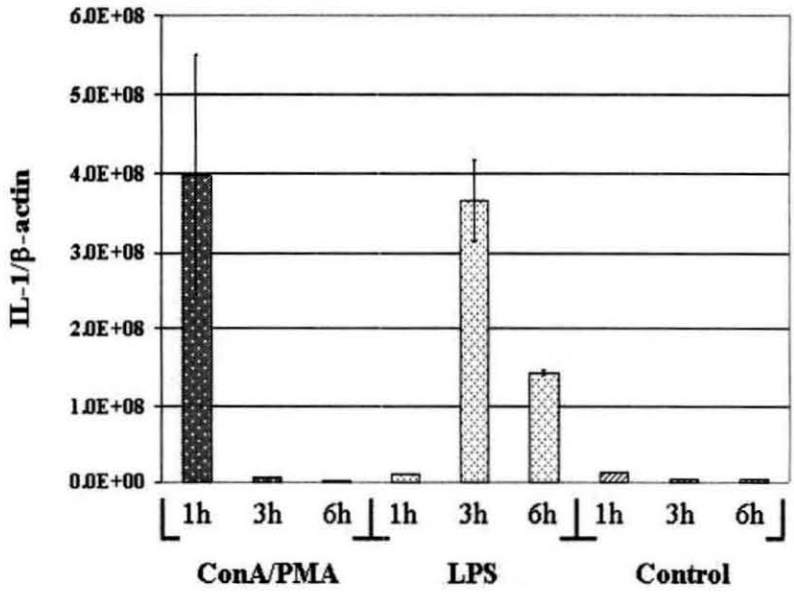


Fig.5 Quantitative real time PCR analysis of IL-1β expression in Japanese flounder following stimulation of PBLs with ConA/PMA or LPS at 1, 3, and 6 hours intervals. Standardization was done with the respective β-actin mRNA levels.



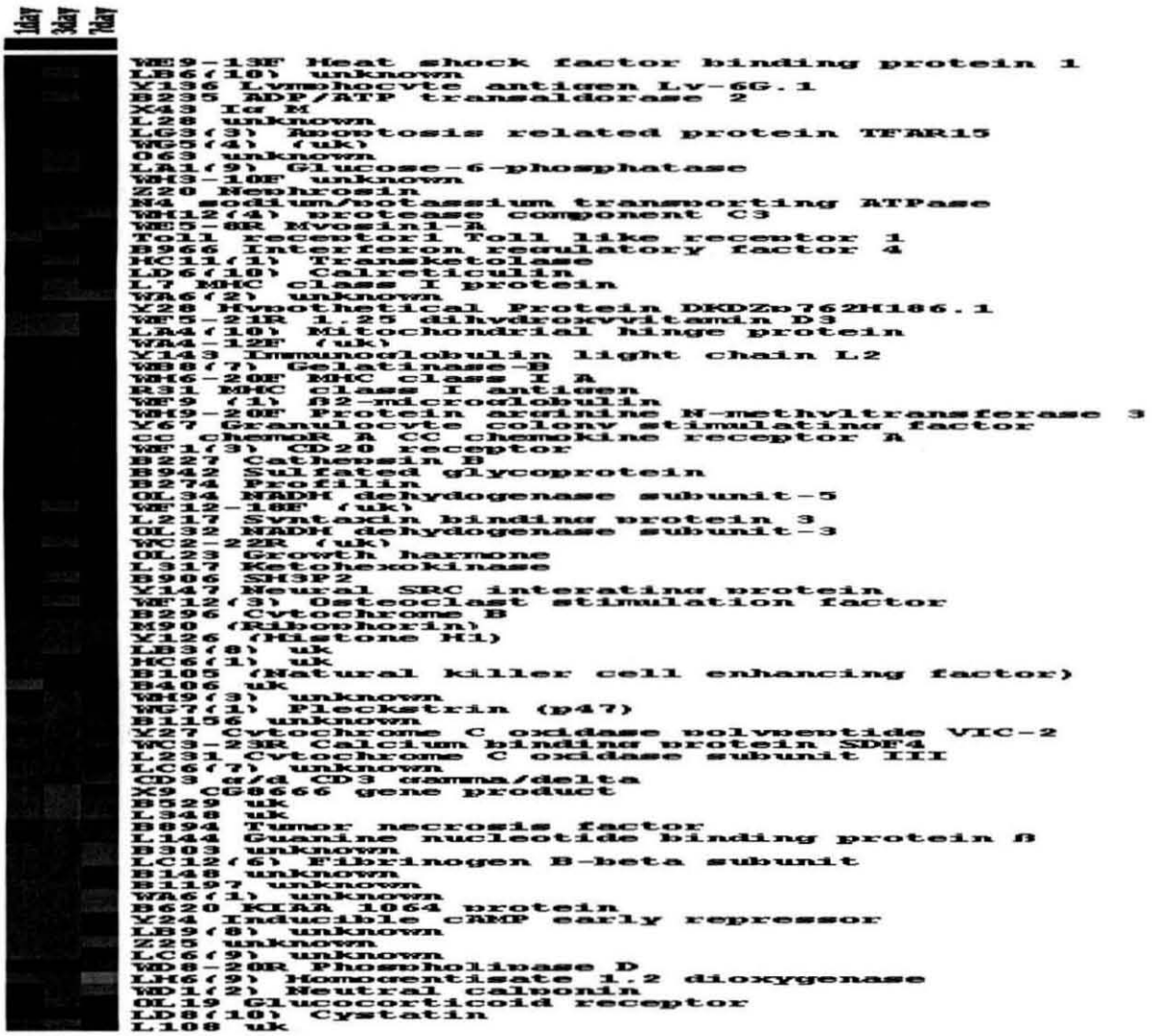


Fig.6 Expression pattern of IL-1β inducible genes expressed in microarrays. Red color indicates up-regulation and green color indicates down-regulation of genes.

Table 2 IL-1β induced gene expression profile of Japanese flounder kidney cells *in vivo*

Cluster No.	Gene name	Clone ID	Gene accession No	Function	Expression levels		
					1day[a]	3day[b]	7day[c]
Cluster 1	Tumor necrosis factor	B894	AU091130	Inflammation	3.4	ND[d]	0.8
(Cytokines)	Granulocyte colony stimulating factor	Y67	AU260798	Granulopoisis	1.2	5	0.7
	CC chemokine receptor A	cc chemoR A	Not submitted	Chemotaxis	1.4	2.7	0.8
Cluster 2	Lymphocyte antigen Ly-6G.1	Y136	AU260850	Cell growth	3.9	4.6	1
(Surface antigens)	Guanine nucleotide binding protein β	L144	AU260989	Receptor coupling	2.7	ND	ND
	MHC class I protein	L7	AU260469	Antigen presenting	2.2	ND	1.1
	CD20 receptor	WF1(3)	C82121	B cell differentiation	1.4	4.2	0.8
	MHC class I A	WH6-20F	AU050612	Antigen presenting	1.8	3.8	1
	β2-microglobulin	WF9 (1)	C81987	Antigen presenting	1.5	3.5	1.1
	MHC class I antigen	R31	AU260597/AU260890	Antigen presenting	1.4	2.2	1
	Ig M	X43	AU260744/AU260976	Antigen presenting	1.9	2	1
	Immunoglobulin light chain L2	Y143	AU260856	Antigen presenting	1.3	0.8	2
	CD3 gamma/delta	CD3 g/d	AB044572	Antigen presenting	ND	0.5	ND
Cluster 3	Pleckstrin (p47)	WG7(1)	C81996	Signal transduction	3.3	ND	0.7
(Signal transduction genes)	Phospholipase D	WD8-20R	AU050587	Signal transduction	1.3	5.1	ND
	Toll like receptor 1	Toll receptor1	AB109394	Signal transduction	ND	1.1	3.2
	SH3P2	B906	AU091137/AU091138	Signal transduction	1.6	ND	2
	Inducible cAMP early repressor	Y24	AU260762	signal transduction	ND	ND	0.5
	KIAA 1064 protein	B620	AU091006	signal transduction	ND	ND	0.3
Cluster 4	Cytochrome C oxidase subunit III	L231	AU260875	Apoptosis	4	ND	0.7
(Apoptosis genes)	Heat shock factor binding protein 1	WE9-13F	AU050269	Apoptosis	2.4	2.4	1.2
	Cytochrome C oxidase polypeptide VIC-2	Y27	AU260765	Apoptosis	4	ND	0.7
	Apoptosis related protein TFAR15	LG3(3)	C23106/C23107	Apoptosis	2.1	1.2	0.9
	Interferon regulatory factor 4	B966	AU091159	Apoptosis	2	0.7	1.2
	Cathepsin B	B227	AU090814	Apoptosis	1.2	7.5	1.1
	Mitochondrial hinge protein	LA4(10)	C23431	Apoptosis	ND	ND	3.2
	Cytochrome B	B296	AU090859	Apoptosis	1.2	1.4	2
	1,25 dihydroxyvitamin D3	WF5-21R	AU050667	Apoptosis	ND	ND	2
	Cytochrome C oxidase subunit-1	OL8	AU260722	Apoptosis	ND	0.2	ND
Cluster 5	Gelatinase-B	WB8(7)	C82368	Metalloproteinase	2.6	5.5	1
(Inflammation genes)	Nephrosin	Z20	AU260535	Metalloproteinase	2.5	1.3	1.6
	protease component C3	WH12(4)	C82226	Innate immunity	3.4	ND	ND
	Natural killer cell enhancing factor	B105	AU090730/AU090731	Innate immunity	0.4	1.7	0.8
	Sulfated glycoprotein	B942	AU091151	Inflammation	1.1	2.9	1.1
	Osteoclast stimulation factor	WF12(3)	C82131/C82132	Inflammation	1.3	ND	2.4
	Homogentisate 1,2 dioxygenase	LH6(9)	C23426	Inflammation	ND	ND	0.2
Cluster 6	ADP/ATP transaldorase 2	B235	AU090822	Metabolism	3.9	ND	1
(Other regulated genes)	Glucose-6-phosphatase	LA1(9)	C23369	Metabolism	3.5	ND	1
	sodium/potassium transporting ATPase	N4	AU260498	Metabolism	2.9	1.3	1.6
	Transketolase	HC11(1)	C23499	Metabolism	2.6	ND	1.1
	Myosin1-A	WE5-8R	AU050053	Protein metabolism	2.3	ND	1.4
	Hypothetical Protein DKDZp762H186.1	Y28	AU260766	Unknown	2.2	0.9	1.1
	Calreticulin	LD6(10)	C23456	Molecular chaperone	2.1	0.9	1.1
	Protein arginine N-methyltransferase 3	WH9-20F	AU050616/AU050617	Methylation	1.5	4.9	1.1
	Ketohexokinase	L317	AU261082	Metabolism	1.3	4	1.5
	Profilin	B274	AU090848	Cell differentiation	0.9	3.4	1.4
	NADH dehydogenase subunit-5	OL34	BAA89043	phosphorylation	1	2.5	1.2

(Continued…)

	Gene name	Clone ID		Function	Expression levels		
					1day	3day	7day
	NADH dehydogenase subunit-3	OL32	BAA89040	phosphorylation	1.6	2.3	1.6
	Growth harmone	OL23	BAA06159	Endocrine metabolism	1.2	2.1	1.2
	Syntaxin binding protein 3	L217	AU261026	Phosphorylation	1.5	2	1.3
	Neural SRC interating protein	Y147	AU260859	Phosphorylation	1.1	2	1.4
	Glycil tRNA ligase	B230	AU090816	Unknown	ND	ND	3.2
	Glycine decorboxylase	LB6(5)	C23180	Metabolism	ND	ND	2.3
	Ribophorin	M90	AU260489	Protein metabolism	0.4	ND	0.4
	Histone H1	Y126	AU260840	DNA packing	0.4	ND	0.4
	CG8666 gene product	X9	AU260729	Unknown	ND	0.3	0.8
	Cystatin	LD8(10)	C23459	Ca+2 binding	0.8	0.7	0.5
	Glucocorticoid receptor	OL19	O73673	Immunosupression	1.1	ND	0.5
	Calcium binding protein SDF4	WC3-23R	AU050772	Ca+2 binding	ND	ND	0.4
	Neutral calponin	WD1(2)	C82034	Cell differentiation	1.2	ND	0.3
	Fibrinogen B-beta subunit	LC12(6)	C23252/C23253	Blood clotting	ND	ND	0.3
Cluster 7	uk[g]	O63	AU260562	Unknown	2.7	ND	0.9
(Unknown genes)	uk	WA6(2)	C82006,C82007	Unknown	2.6	ND	ND
	uk	LB6(10)	C23439/C23440	Unknown	2.3	ND	1.2
	uk	WH9(3)	C82149/C82150	Unknown	2.3	ND	0.7
	uk	WH3-10F	AU050166	Unknown	2.3	0.8	1
	uk	L28	AU050541	Unknown	2.2	1.5	1.3
	uk	WC2-22R	AU050705	Unknown	2.1	ND	2.2
	uk	WF12-18F	AU050518	Unknown	2	ND	1.7
	uk	WG5(4)	C82212/C82213	Unknown	2	1.2	0.9
	uk	WA4-12F	AU050212	Unknown	2	0.8	2.2
	uk	B114	AU090735	Unknown	1.5	2	2.1
	uk	WE8(3)	C82115	Unknown	1.3	2.4	0.5
	uk	WC12-12R	AU050230	Unknown	1.1	2.2	1
	uk	WA11-10R	AU050134	Unknown	1	3.2	1.1
	uk	WC2-9R	AU050090	Unknown	1	3.1	ND
	uk	WF1-21F	AU050662	Unknown	ND	ND	3.7
	uk	LB3(8)	C23331	Unknown	0.4	ND	0.5
	uk	HC6(1)	C23496/C23497	Unknown	0.4	1.6	0.5
	uk	B406	AU090904	Unknown	0.3	1.3	1
	uk	B529	AU090990	Unknown	ND	0.3	ND
	uk	L348	AU261109	Unknown	ND	0.3	ND
	uk	L108	AU260964	Unknown	ND	0.3	1
	uk	LB9(8)	C23335	Unknown	ND	ND	0.5
	uk	B148	AU090760	Unknown	ND	ND	0.5
	uk	B1197	AU091263	Unknown	ND	ND	0.5
	uk	LC6(9)	C23384	Unknown	ND	ND	0.5
	uk	LC6(7)	C23300/C23301	Unknown	ND	ND	0.4
	uk	B1156	AU091232	Unknown	ND	ND	0.4
	uk	Z25	AU260880	Unknown	ND	ND	0.3
	uk	WA6(1)	C81936/C81937	Unknown	ND	ND	0.3
	uk	B303	AU090862	Unknown	ND	ND	0.3

[a] IL-1β induced gene expression level after 1day
[b] IL-1β induced gene expression level after 3 day
[c] IL-1β induced gene expression level after 7 day
[d] ND means not detected
[e] Up-regulated gene expression level indicated in dark shades (> 2.0)
[f] Down-regulated gene expression level indicated in light shades(<0.5)
[g] uk means unknown

As expected, JF IL-1β was not found in the non stimulated leucocytes. In contrast, in gilt head seabream, IL-1β transcript was found in some of the tissues.[17] In our study, the maximum level of IL-1β was observed 3 h after LPS stimulation. Similarly, IL-1β expression in rainbow trout significantly increased between 2.5 and 4 hours after stimulation with LPS.[18] Understanding the expression of immune response genes will help to characterize disease-related inflammatory pathways and to identify the functional properties of immune cell subpopulations. Therefore, an accurate quantification of mRNA expression is needed to assess differential gene expression. The greatest increase in the copy number of IL-1β mRNA was 30 fold after stimulation with ConA/PMA (Fig. 3).

In this study, we also focused on the gene expression pattern of immune-related genes of Japanese flounder kidney cells induced by IL-1β *in vivo* as kidney is the major lymphoid organ in fish and IL-1β is a potential inflammatory molecule. Tucker et al. reported detection of bombarded CAT (reporter gene) expression in fish of the CMV promoter group and fish of the SV-40 promoter group after plasmid DNA injection. The CAT expression in the fish group injected with CMV promoter was higher than that in the fish group injected with SV-40 promoter.[19] Kono et al. studied the IL-1β protein expression in serum on 1, 3, and 5 days after pCMV-IL-1β plasmid injection.[20] Therefore in this stdudy we used a CMV promoter –driven vector.

The immune-related genes that were spotted on the microarray glass slide were related to inflammation, cell proliferation, signaling pathways, cell surface molecules, transcription factors and apoptosis. In our study, IL-1β induced gene expression was highest on day 1 over day 3 and day 7 after injection (Table.2). Our results are in

complete agreement with the findings of Kono et al., in which the expression of IL-1β was highest on day 1 over day 3 and day 5 after injection.[20] The present results show that IL-1β induced the expression of Tumor necrosis factor (TNF), Granulocyte colony stimulating factor (G-CSF) and chemokine receptor A (Table. 2). A typical characteristic of IL-1β is to synergize with a variety of cytokines including TNF and G-CSF. Synergism between IL-1β and TNF has also observed *in vivo*.[21] Endotoxin or secondary mediators such as TNF, IL-1β and IFN-γ are major stimulators of G-CSF production *in vivo* and result in rapid but transient elevation in serum.[22] Our results of IL-1β-induced TNF and G-CSF *in vivo* agree with the findings of other research group. The induction of chemokine receptor A in our study indicates that IL-1β acts as a chemo attractant, as previously reported.[23]

IL-1β plays a central role in host defense. It induces differentiation and expression of surface immunoglobulin in pre B cells, proliferation of Th2 cells in combination with stimulation through the TCR, and proliferation and immunoglobulin secretion of mature B cells.[24] Our finding that JF IL-1β induced the expressions of IgM, CD20, β-2-microglobulin and the immunoglobulin light chain (Table. 2) suggests that it has a role in humoral immunity. CD20 also has a role in B cell activation and differentiation.[25] The increase in the expression of MHC class I antigens in our study raises the possibility that IL-1β acts as a mediator of cellular immunity. This is because the adjuvant effect of IL-1β requires increased cytotoxic lymphocyte activity.[26] CD3 gamma/delta plays a role in TCR complex assembly and in signal transduction mechanism.[27] Surprisingly, CD3 gamma/delta was down regulated in our experiment.

IL-1 β in mammals is reported to be a signaling molecule for communication between the immune system and other internal systems.[28] Toll-like receptor 1 (TLR-1), SH3P2, and Pleckstrin 47 were induced in response to IL-1β in our study (Table. 2). TLR-1 is expressed ubiquitously and at rather high levels. It associates with and regulates the TLR-2 response. TLR-2 activates the NF-kB pathway, which regulates cytokine expression through MyD88. Activation of the NF-kB pathway initiates the adaptive immune response by producing inflammatory cytokines such as IL-1, IL-6, IL-8 and IL-12.[29] SH3P2 protein (28 kDa) has a short praline-rich stretch and a SH3 domain at the amino terminus, followed by three ankyrin repeats. Proteins containing SH3 domains are essential in several well characterized signaling pathways, including the Ras/MAPK pathway, which is involved in cellular division, differentiation and cytoskeleton reorganization in response to growth factor receptor activation. Interaction of SH3P2 with Cb1 induced Src-mediated tyrosine phosphorylation of SH3P2.[30] Transcription factors that activate IL-1β gene expression include NF-kB, AP-1 and a tyrosine phosphorylated protein.[31] We believe that IL-1β-induced SH3P2 plays a role in tyrosine phosphorylation and MAPK signal transduction pathway. cAMP pathways are involved in regulating transcription of IL-1β.[32] cAMP inducing agents and IL-1 itself have been shown to sustain m RNA levels for over 24 hours in human blood monocytes.[33] In this study, as expected, IL-1β down regulated inducible cAMP early repressor (Table. 2).

IL-1β performs a variety of roles, acting sometimes as pro-apoptotis factor and some times as an anti-apoptosis factor. In the latter case, it protects against TNF-mediated cell death by arresting cell cycling.[34] IL-1 is processed and released during apoptosis.[35] Cytochrome c oxidase is a pro apoptosis molecule. Several apoptotic

pathways release cytochrome c from the mitochondrial intermembrane space, resulting in the activation of downstream caspases.[36] IRF4 activates the basal transcription of IL-1 beta promoter in macrophages and fibroblasts [37] and is essential for the function of B cells and cytotoxic T lymphocytes.[38] Cathepsin B has different roles. It protects cytotoxic lymphocytes from self destruction after degranulation.[39] TRAF15 is an apoptosis-related protein according to entries in the databases. Cytochrome b is an essential component of the super oxide-generating oxidase.[40] Our results seem to show that IL-1β activates both pro and anti apoptosis molecules.

Many other genes with different functions were also induced in response to IL-1β, such as matrix metalloproteinases (MMP) (e.g., gelatinase) and oxidative phosphorylation-related NADH dehydrogenases. MMP genes are strongly induced by IL-1β.[7] IL-1 has been shown to up-regulate monocyte and macrophage MMP (gelatinase) production *in vitro*.[41] As expected, the glucocorticoid receptor was down regulated in our study (Table. 2). Corticosteroids inhibit the transcription of IL-1. Increased synthesis of 1kB with decreased translocation of NF-kB is thought to account for the suppressive effect of glucocorticoids on cytokine synthesis.[42] Some unknown genes were up or down regulated in our experiment. In our previous EST analyses 35-40% of the genes were unknown genes which did not show any homology to the known genes in the gene bank databases. Some of these genes are expected to have immune function. The nucleotide sequencing and protein expression patterns are expected to give more complete information about these genes.

In conclusion, our results demonstrate that the immune response in Japanese flounder is significantly influenced by IL-1β, both *in vitro* and *in vivo*. The fish injected

with IL-1β cDNA using a DNA injection method stimulated the expression of immune related genes, suggesting that Japanese flounder IL-1β gene has functional similarity with that of mammalian IL-1β. Dissociation between transcription and translation is characteristic of IL-1β.[43] The microarray analysis made it possible to identify these immune responses at the transcriptional level. Further studies are needed to understand the changes at the translational level.

ACKNOWLEDGEMENTS

This research was supported in part by the Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology and Research Project for utilizing advanced technologies in Agriculture, Forestry and Fisheries of Japan.

2.6. REFERENCES

1. Hughes AL. Evolution of the interleukin-1 gene family in mammals. *J. Mol. Evol.* 1994; **39**: 6-12
2. Dinarello CA. Interleukin-1. *Cytokine Growth Factor. Rev.* 1997; **8**: 253-265.
3. Fujiki K, Shin DH, Nakao M, Yano T. Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1β, high affinity Immunoglobulin E Fc-receptor γ subunit and serum amyloid A. *Fish Shellfish Immunol.* 2000; **10**: 229-242.
4. Scapigliati G, Buonocore F, Bird S, Zou J, Pelegrin P, Falasca C, Prugnoli D, Secombes CJ. Phylogeny of cytokines: molecular cloning and expression analysis

of seabass *Dicentrarchus labrax* interleukin-1β. *Fish Shellfish Immunol.* 2001; **11**: 711-726.

5. Dhiman N, Bonilla R, O'Kane DJ, Poland GA. Gene expression microarrays: a 21st century tool for directed vaccine design. *Vaccine* 2002; **20**: 22-30.
6. Young RA. Biomedical discovery with DNA arrays. *Cell* 2000; **102**: 9-15.
7. Vincenti MP, Brinckerhoff CE. Early response genes induced in chondrocytes stimulated with the inflammatory cytokine interleukin-1β. *Arthritis Res.* 2001; **3**: 381-388.
8. Hirono I, Nam BH, Kurobe T, Aoki T. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder, *Paralicthys olivaceus. J. Immunol.* 2000; **165**: 4423-4427.
9. Felsenstein J. PHYLIP (phylogeny inference package), Version 4.0. Department of Genetics, University of Washington, Seattle 1996.
10. Sambrook J, Russell DW. Molecular Cloning A Laboratory Manual, 3rd ed. 2001; Cold Spring Harbor Laboratory Press, New York.
11. Park CI, Kurobe T, Hirono I, Aoki T. Cloning and characterization of cDNAs for two distinct tumor necrosis factor receptor superfamily genes from Japanese flounder *Paralichthys olivaceus. Dev. Comp. Immunol.* 2003; **27**: 365-375.
12. Katagiri T, Hirono I, Aoki T. Identification of a cDNA for medaka cytoskeletal β- actin and construction for the reverse transcriptase-polymerase chain reaction (RT-PCR) primers. *Fish. Sci.* 1997; **63**: 73-76.

13. Kurobe T, Yasuike M, Kimura T, Hirono I, Aoki T. Expression profiling of Japanese flounder *Paralichthys olivaceus* immune-related genes using cDNA microarrays. *Dev. Comp. Immunol.* 2005 (in press).

14. Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A. Identification of a common nucleotide sequence in the 3'- untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Natl. Acad. Sci. USA*. 1986; **83**: 1670-1674.

15. Thonrberry NA, Bull HG, Calacay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J. A novel heterodimeric cystein protease is required for interleukin-1 beta processing in monocytes. *Nature* 1992; **356**: 768-774.

16. Miyamoto S, Kimball SR, Safer B. Signal transduction pathways that contribute to increased protein synthesis during T-cell activation. *Biochem. Biophys. Acta.* 2000; **1494**: 28-42.

17. Pelegrin P, Castillo JG, Mulero V, Meseguer J. Interleukin-1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines. *Cytokine* 2001; **16**: 67-72.

18. Zou J, Holland J, Pleguezuelos O, Cunnigham C, Secombes CJ. Factors influencing the expression of interleukin-1β in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. *Dev. Comp. Immunol.* 2000; **24**: 575-582.

19. Tucker C, Endo M, Hirono I, Aoki T. Assessment of DNA vaccine potential for juvenile Japanese flounder, *paralichthys olivaceus*, through the introduction of

reporter genes by particle bombardment and histopathology. *Vaccine* 2001; **19**: 801-809.

20. Kono T, Fujiki K, Nakao M, Yano T, Endo M, Sakai M. The immune responses of common carp, *Cyprinus carpio L.*, injected with carp interlekin-1β gene. *J. Interferon Cytokine Res.* 2002; **22**: 413-419.
21. Dinarello CA. Biological basis for interleukin-1 in disease. *Blood* 1996; **87**: 2095-2147.
22. Basu S, Dunn A, Ward A. G-CSF: Function and modes of action (review). *Int. J. Mol. Med.* 2002; **10**: 3-10.
23. Taub DD. Chemokine-leukocyte interactions. The voodoo that they do so well. *Cytokine Growth Factor Rev.* 1996; **7**: 355-376.
24. Yin Z, Kwang J. Carp interleukin-1β in the role of an immuno-adjuvant. *Fish Shellfish Immunol.* 2000; **10**: 375-378.
25. Uchida J, Lee Y, Hasegawa M, Liang Y, Baradney A, Oliver JA, Bowen K, Steeber DA, Haas KM, Poe JC, Tedder TF. Mouse CD20 expression and function. *Int. Immunol.* 2003; **16**: 119-129.
26. Kiertscher SM, Mathews HL. Systemic IL-1 and adjuvant treatment of an experimental tumor. II. Immune status during primary tumor challenge. *Biotherapy* 1992; **5**: 259-274.
27. Lai L, Alaverdi N, Maltais L, Morse III HC. Mouse cell surface antigens: nomenclature and immunophenotyping. *J. Immunol.* 1998; **160**: 3861-3868.

28. Engelsma MY, Stet RJM, Schipper H, Verburg-van Kemenade BML. Regulation of interleukin-1β RNA expression in the common carp, *Cyprinus carpio L. Dev. Comp. Immunol.* 2001; **25**: 195-203.

29. Opal SM, Huber CE. Bench-to-bedside review: Toll-like receptors and their role in septic shock. *Critical Care* 2002; **6**: 125-136.

30. Vidal M, Gigoux V, Garbay C. SH2 and SH3 domains as targets for anti-proliferative agents. *Critical Rev. Oncol/Heamatol.* 2001; **40**: 175-186.

31. Reddy S, Devlin R, Menaa C, Nishimura R, Choi SJ, Dallas M, Yoneda T, Roodman GD. Isolation and characterization of a cDNA clone encoding a novel peptide (OSF) that enhances osteoclast formation and bone resorption. *J. Cell. Physiol.* 1998; **177**: 636-645.

32. Curfs JHA, Meis JFGM, Hoogkamp-Korstanje JAA. A primer on cytokines: sources, receptors, effects, and inducers. *Clinical Microbiol. Rev.* 1997; **10**: 742-780.

33. Serkkola E, Hurme M. Synergism between protein-kinase C and cAMP dependent pathways in the expression of the interleukin 1β gene is mediated via the activaror- protein-1(AP-1) enhacer activity. *Eur. J. Biochem.* 1993; **213**: 243-249.

34. Belizario JE, Dinarello CA. Interleukin1, interleukin 6, tumor necrosis factor and transforming growth factor β increase cell resistance to tumor necrosis factor cytotoxicity by growth arrest in the G1 phase of the cell cycle. *Cancer Res.* 1991; **51**: 2379-2385.

35. Hogquist KA, Nett MA, Unanue ER, Chaplin DD. Interleukin is processed and released during apoptosis. *Proc. Natl. Acad. Sci. USA.* 1991; **88**: 8485-8489.

36. Mootha VK, Wei MC, Buttle KF, Scorrano L, Panoutsakopoulou V, Mannella CA, Korsmeyer SJ. A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. *EMBO J.* 2001; **20**: 661-671.

37. Marecki S, Atchison ML, Fenton MJ. Differential expression and distinct functions of IFN Regulatory Factor-4 and IFN consensus sequence binding protein in macrophages. *J. Immunol.* 1999; **163**: 2713-2722.

38. Lohoff M, Mittrücker, H-W, Prechtl S, Bitchoff S, Sommer F, Kock S, Ferrick DA, Duncan GS, Gassner A, Mak TW. Dysregulated T helper cell differentiation in the absence of Interferon regulatory factor 4. *Proc. Natl. Acad. Sci. USA.* 2002; **99**: 11808-11812.

39. Balaji KN, Schaschke N, Machleidt W, Catalfamo M, Henkart PA. Surface cathepsin B protects cytotoxic lymphocytes from self-destruction after degranulation. *J. Exp. Med.* 2002; **196**: 493-503.

40. Newburger PE, Dai Q, Whitney C. *In vitro* regulation of human phagocyte cytochrome b heavy and light chain gene expression by bacterial lipopolysaccharide and recombinant human cytokines. *J. Biol. Chem.* 1991; **266**: 16171-16177.

41. Saren P, Welgus H, Kovanen PT. TNF-α and IL-1β selectively induce expression of 92-kDa gelatinase by human macrophages. *J. Immunol.* 1996; **157**: 4159-4165.

42. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: Inhibition of NF-kB activity through induction of IkB synthesis. *Science* 1995; **270**: 286-290.

43. Schindler R, Clark BD, Dinarello CA. Dissociation between interleukin-1β mRNA and protein synthesis in human peripheral blood mononuclear cells. *J. Biol. Chem.* 1990; **265**: 10232-10237.

Chapter 3

Adjuvant effects of IL-1β, IL-8 homologue and JFCC1 on DNA vaccination against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) and analysis of chemokines (IL-8, JFCC1) immune response using cDNA microarrays

ABSTRACT: Three fish cytokine genes (IL-1β, IL-8 and JFCC1) were evaluated for their adjuvant effect on a suboptimal dose of viral hemorrhagic septicemia virus DNA vaccine carrying the glycoprotein gene (VHS g) in Japanese flounder, *Paralichthys olivaceus*. Fish were intramuscularly injected with 0.1 μg of VHS g vaccine plus a 10 μg of cytokine expression plasmid. Experimental challenge of 1 X 10^3 $TCID_{50}$ virus eight days post-vaccination revealed that the adjuvants used in this study were able to provide significant protective immunity against VHS and this lasted until 21 days after challenge. The adjuvant vaccines had a high protective efficiency, giving relative percentage survival (RPS) values of at least 70 percent. The defense mechanisms activated by two of the cytokines (IL-8, JFCC1) were further elucidated by microarray analysis. IL-8 up-regulated specific immune-related genes such as Ig M, Ig D, MHC class IA and T cell receptor β at 1 day post-injection. JFCC1 up-regulated non-specific and specific immune-related genes such as IL-8, CC chemokine receptor A, lysozyme, Ig M and β2-microglobulin. Our results confirm that the adjuvants elicit strong humoral and cellular immune responses which may play a pivotal role in protecting the fish during virus infections.

KEYWORDS: **Japanese flounder; Viral haemorrhagic septicemia virus (VHSV); DNA vaccine; Adjuvants; Microarrays; Gene expression profiling; Immune-related genes.**

3.1. INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is the most serious viral pathogen affecting fresh water and marine species.[1] In 1999, VHSV infection occurred in wild Japanese flounder, *Paralichthys olivaceus*, in Japan.[2] Recently, novel vaccination strategies using antigen–encoding DNA plasmids have been shown to successfully induce protective cellular and humoral immune responses against a variety of infectious diseases in fish, including VHSV in Japanese flounder.[3] Various strategies have been used to increase the efficiency of DNA vaccination. For example, immune responses induced by DNA vaccination can be enhanced by co-injection with recombinant cytokines or plasmids encoding these cytokines.[4]

Utilization of cytokines as adjuvants for fish viral diseases is becoming more feasible as fish homologues of mammalian cytokine genes become available.[5] Several Japanese flounder genes homologous to mammalian cytokines have been cloned including IL-1β,[6] IL-8[7] and CC chemokines.[8] Recently we constructed a cDNA chip that contains approximately 900 different cDNA clones including 91 immune-related genes.[9] Microarrays can be used to study the response of a host to challenge with the stimulant such as cytokine and identify immune gene responses.[10] A microarray can be a useful tool for quantitatively analyzing the expression of innate, humoral and cellular immune-related molecules following DNA vaccine and expression plasmid injection in fishes.[3, 6]

In the present study, a panel of plasmids expressing three cytokine genes, *Viz.* IL-1β, IL-8 homologue and JFCC1 of Japanese flounder, were evaluated for adjuvant effect on vaccination with a recombinant plasmid expressing the VHSV glycoprotein gene in Japanese flounder. Using microarray analysis, we studied the expression profile of genes

that are responsible for non-specific and specific immune responses at 1, 3 and 7 days following injection of IL-8 homologue and JFCC1 expression plasmids.

3.2. MATERIALS AND METHODS

3.2.1. Cells and virus

VHSV-KRRV strain 9822, which was isolated from farmed Japanese flounder in Kagawa prefecture in Japan,[11] was kindly provided by Dr. T. Isshiki of the Kagawa Prefectural Fisheries Experimental Station. The virus was propagated in hirame natural embryo (HINAE) cell line.[12] For virus propagation, HINAE cells were infected with virus and incubated until a cytopathic effect (CPE) was observed. The supernatant was collected by centrifugation at 2500 X g for 5 min and stored in 1-ml aliquots at -80 ^{0}C until use.

3.2.2. Construction of recombinant pCMV-VHSg vaccine and cytokine expression plasmids

pCMV-VHSg vaccine[3] was constructed by cloning the VHS glycoprotein gene into the pCDNA 3.1(+) expression plasmid (Invitrogen, USA). The pCMV-IL-1β expression plasmid was cloned into pCI-neo mammalian expression vector (Promega, Madison, WI, USA) as described earlier.6 cDNAs for a full-length Japanese flounder IL-8 homologue and JFCC1 were cloned into pCI-neo mammalian expression vector (Promega, Madison, WI, USA) following the manufacturer's instructions, and named pCMV-IL-8 and pCMV-JFCC1 respectively. The expression plasmids were extracted by ultracentrifugation using a CsCl-ethidium bromide gradient.[13]

3.2.3. Vaccination with pCMV-VHSg and cytokine expression plasmids

Japanese flounder juveniles weighing approximately 3 g were given 50 µl intramuscular injections containing 0.1 µg and 1µg of pCMV-VHSg plus 10 µg cytokine gene plasmids in phosphate buffered saline (PBS). Fish in the negative control group were each injected with 50 µl PBS. The positive controls were injected with pCMV-VHS g (0.1 µg and 1µg) or cytokine expression plasmids (10 µg) alone. The fish were fed daily and the water temperature was kept at 12-13 ^{0}C in a re-circulated water system.

3.2.4. Sampling

Kidney tissue was collected from six fish from each of the three groups (IL-8 homologue group, JFCC1 group and empty vector group) at 1, 3 and 7 days post-immunization and stored at -80 ^{0}C until total RNA extraction for microarray analysis.

3.2.5. Challenge experiments

Eight days after vaccination, fish were experimentally challenged with VHSV by intraperitoneal injection. Thirty fish from each group were given a dose of 1 X 10^{3} $TCID_{50}$. Cumulative mortality was monitored daily over a three-week period.

3.2.6. Statistical analysis

Differences between the experimental and control groups were analyzed by $\chi 2$ test with Yates' correction. Relative percentage survival (RPS) was determined by the formula **RPS** = (1- % mortality of vaccinated fish/ % mortality of control fish) X 100.[14]

3.2.7. Microarray analysis

The cDNA microarray chip used in this study contained a total of 871 clones consisting of 91 immune-related genes, 371 genes of unknown function and 409 genes of

metabolism-related and housekeeping genes.[9] Each gene was spotted in duplicate to facilitate comparison during the analysis.

Total RNA was isolated from kidney cells from six fish in each of the three groups at 1, 3 and 7 days post-immunization using Trizol (Invitrogen Life technologies, USA). A sample of total RNA sample (50μg) from each group was synthesized into cDNA using a Labelstar Array Kit (Qiagen, Japan) and the purified cDNAs were labeled with Cy3 or Cy5-dCTP using a Cyscribe First-Strand cDNA Labeling Kit (Amersham Biosciences, USA) following the manufacturer's instructions. The arrayed clones were hybridized with an equal volume of Cy3- or Cy5-labeled first strand cDNA in hybridization buffer. Hybridization was carried out at 42 ^{0}C for 18 h. After 18 h, washing was performed with 2X SSC-0.1% SDS for 20 min at room temperature, 0.2X SSC-0.1% SDS for 20 min at room temperature, two washes 0.2X SSC-0.1% SDS for 20 min at 55 ^{0}C with shaking, followed by a rinse with 0.2X SSC-0.1% SDS at room temperature. The glass slides were dried and scanned immediately using a GenePix 4000B scanner (Axon Instruments, USA).

Images obtained from scanning were analyzed by GenePix Pro ver.4.0 software (Axon Instruments, USA). Cy3 and Cy5 signal intensities were normalized to the signal intensity of the spotted housekeeping gene (β-actin) by adjusting the PMT power and signal gain. The signal intensity was calculated as the mean intensity of duplicate spots minus the background signal. The feature ratio was calculated from the stimulated sample signal intensity divided by the control signal intensity. Genes with feature ratios over 2.0 were considered as up-regulated and genes with feature ratios less than 0.5 were considered as down-regulated.

3.2.8. Conventional RT-PCR

Total RNA (2.5μg) from the same sample used for the microarray was reverse transcribed to cDNA with an AMV reverse transcriptase first-strand cDNA synthesis kit (Life Sciences, USA). PCR was performed following initial denaturation at 95 ^{0}C for 5 min, followed by 30 cycles of denaturation at 95 ^{0}C for 30 s, annealing at 55 ^{0}C for 30 s, and elongation at 72 ^{0}C for 1 min, and a final extension step at 72 ^{0}C for 5 min. The primers used are given in Table.1. The PCR products were electrophoresed and visualized in a 1.5% agarose gel using a densitometer (ATTO Co., Japan).

Table.1 Primers used in RT-PCR analysis

Clone	Accession no.	Gene	Sense primer	Antisense primer	Cycle
B120	AU090737	β-actin	5'-TGATGAAGCCCAGAGCAAGA-3'	5'-CTCCATGTCATCCCAGTTGG-3'	25
B1153	AU091230	IL-8	5'-ATCGTTGTTGCTGTGATGGTG-3'	5'-CTCTTAATGTAGCGGCCGATG-3'	30
O26	AU260532	TCR-β	5'-GCACCATTCACACACTGTGGTT-3'	5'-AACAGGCTGGTTTGTGAGCTG-3	30

3.2.9. Expression pattern of inducible genes in microarray analysis

Expression pattern of inducible genes in microarray analysis was analyzed by Cluster 3.0 software.

3.3. RESULTS

3.3.1. Effect of pCMV-VHS g vaccine co-injected with cytokine genes against VHS virus

The cumulative mortality of the VHS g-vaccinated group (I) following challenge with 1 X 10^3 $TCID_{50}$ virus concentration was 48% compared with 23-30% mortality for the adjuvant-vaccinated groups (II, III and IV). On the other hand, 100% of PBS-injected fish (group IX) and 90-93% of the cytokine expression plasmid-injected fish (groups V, VI,

and VII) died following the viral challenge (Fig.1). On the other hand, no adjuvant effect was observed in 1 μg VHS g vaccine (Fig.2).

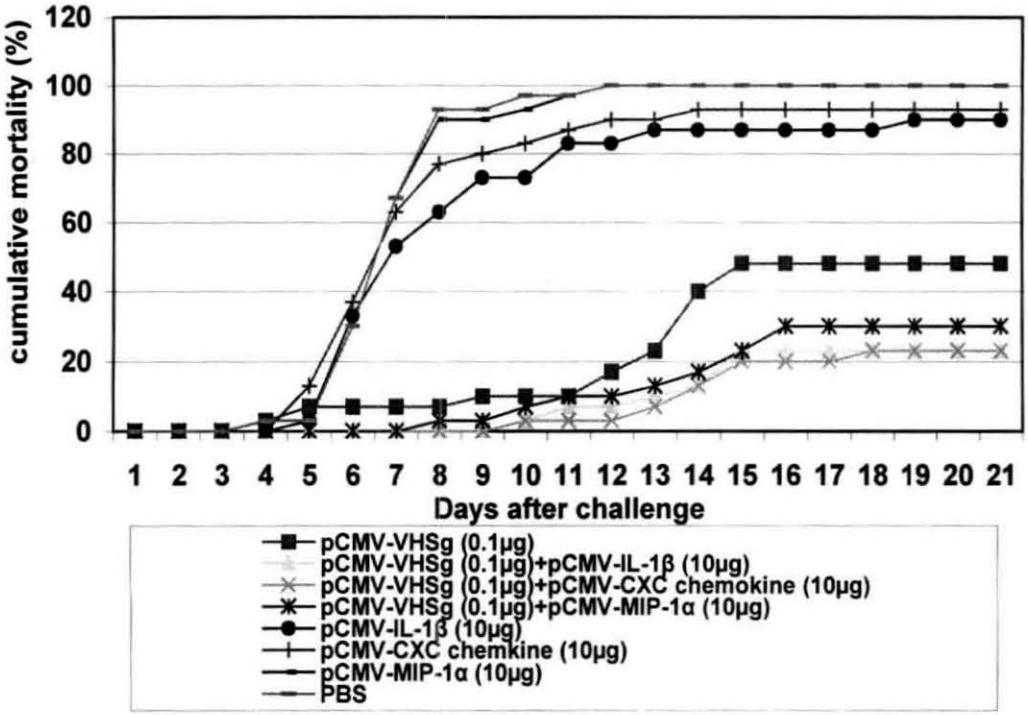


Fig.1 Cumulative mortality of adjuvant vaccinated Japanese flounder challenged with 1 X 10^3 $TCID_{50}$ VHSV/fish. Fish were vaccinated by intramuscular injection and virus challenge was performed eight days post-vaccination by intraperitoneal injection.

Fig.2

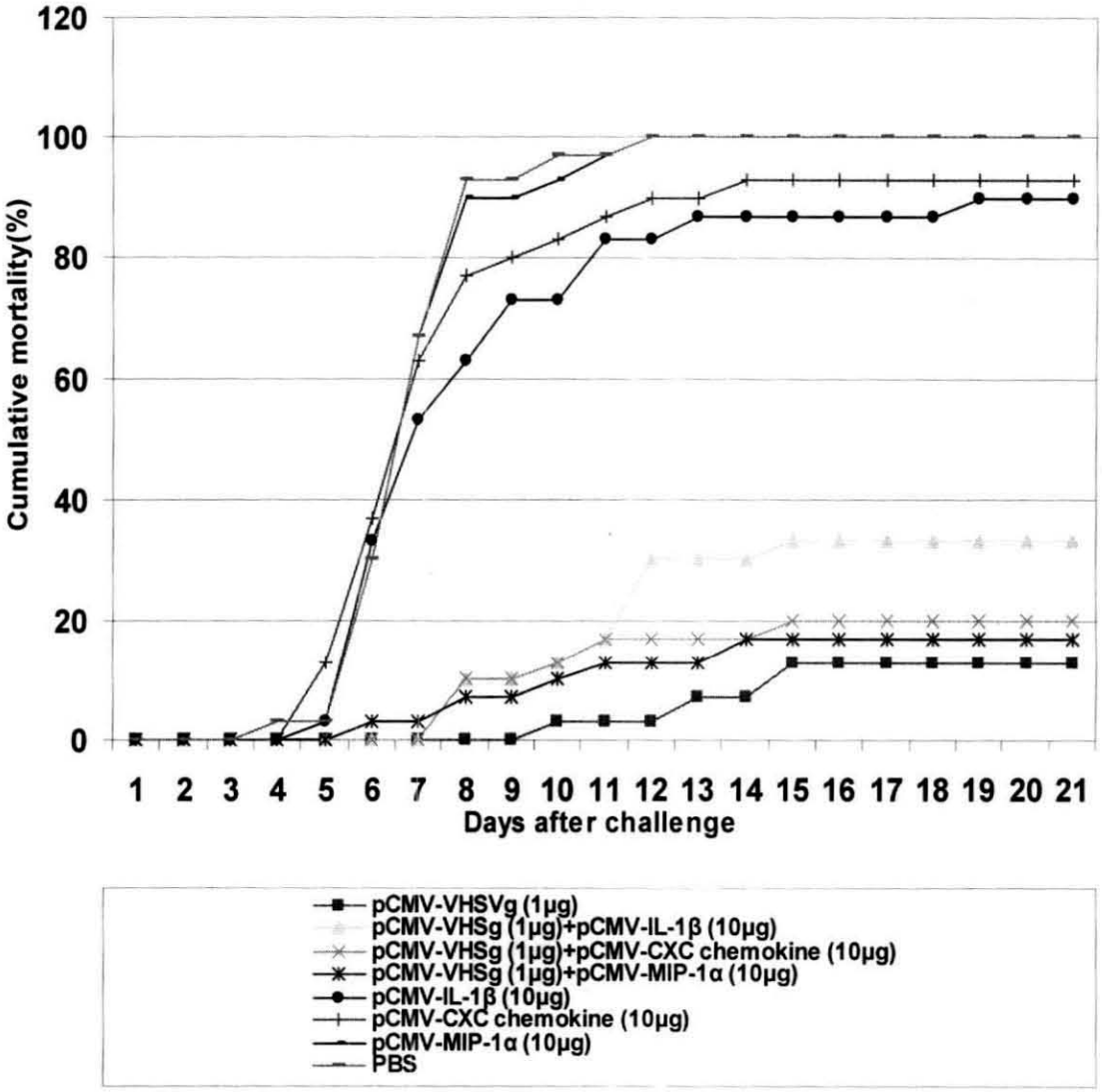


Fig.2 Cumulative mortality of adjuvant vaccinated Japanese flounder challenged with 1 X 10^3 $TCID_{50}$ VHSV/fish. Fish were vaccinated by intramuscular injection and virus challenge was performed eight days post-vaccination by intraperitoneal injection.

As shown in Table.2 the relative percentage survival (RPS) obtained in the VHS g-vaccinated group (I) was 52%; whereas, the adjuvant-vaccinated groups (II, III and IV) had values of 70-77% RPS. The RPS obtained in the cytokine expression plasmid groups (V, VI and VII) was only 7-10% Vs 0% survival in the PBS-injected fish (IX). The Adjuvant-vaccinated groups displayed significantly enhanced survival rates relative to the VHS g-vaccinated group (Table.2). No significant difference was noted in the survival rates among the cytokine expression plasmid groups (Table.2). The RPS values of adjuvants in 1 μg VHS g group (VI, VII, VIII) did not exceed the RPS values of VHS g vaccine 1 μg (V)alone (Table.2) .

Table.2

Experimental group	**$1x10^3$ TCID 50/ fish**		
Adjuvants treatment	**Dead fish/vaccinated (dead fish%)**	**RPS**	**κ^2**
I. pCMV-VHSg (0.1μg)	14/29 (48%)	52	18.16
II. pCMV-IL-1β (10μg)+pCMV-VHSg (0.1μg)	7/30 (23%)	77	34.12
III. pCMV-CXC chemokine (10μg)+pCMV-VHSg (0.1μg)	7/30 (23%)	77	34.12
IV. pCMV-MIP-1α (10μg)+pCMV-VHSg (0.1μg)	9/30 (30%)	70	29.3
V. pCMV-VHSg (1μg)	4/30 (13%)	87	42.42
VI. pCMV-IL-1β (10μg)+pCMV-VHSg (1μg)	10/30 (33%)	67	27.07
VII. pCMV-CXC chemokine (10μg)+pCMV-VHSg (1μg)	6/30 (20%)	80	36.73
VIII. pCMV-MIP-1α (10μg)+pCMV-VHSg (1μg)	5/30 (17%)	83	39.49
IX. pCMV-IL-1β (10μg)	27/30 (90%)	10	N.S
X. pCMV-CXC chemokine (10μg)	28/30 (93%)	7	N.S
XI. pCMV-MIP-1α (10μg)	30/30 (100%)	0	N.S
XII. PBS	30/30 (100%)	0	–

Table.2 Cumulative percentage mortalities and calculated RPS values following challenge with VHSV.

3.3.2. Expression of immune-related genes in kidney cells following IL-8 expression plasmid injection

Several genes were highly expressed 1 day after expression plasmid injection in the IL-8 group (Table.3). These were grouped into humoral immunity, cellular immunity,

other immune-related genes, metabolism-related genes and unknown genes. Some of the important genes that were highly expressed were Ig M, Ig D, MHC class IA, T-cell receptor β (TCR β) and transcription factor BTF3. The expressions of a number of genes in IL-8-induced kidney cells changed at different times after expression plasmid injection (Table.4). The greatest number of genes with changed expression levels (32; 3.7%) was observed 1 day after plasmid injection.

3.3.3. RT-PCR analysis of IL-8- inducible genes

The expression of TCR β greatly increased at 1 day after IL-8 plasmid injection (Fig.3), which strongly supports the results of microarray.

3.3.4. Expression pattern of IL-8 inducible genes

The expression of inducible genes was high on the 1 day followed by 3 day and 7 day (Fig.4).

3.3.5. Expression of immune- related genes in kidney cells following JFCC1 expression plasmid injection

Several genes were highly expressed 3 day after expression plasmid injection in the JFCC1 group (Table.5). The genes were grouped into innate immunity, humoral immunity, other immune-related genes, metabolism-related genes and unknown genes. Some of the important genes that were highly expressed were IL-8, Ig M, Complement component C3 and Lysozyme II. The greatest number of genes with changed expression levels (36; 4.1%) was observed 1 day after plasmid injection (Table.6).

3.3.6. RT-PCR analysis of JFCC1- inducible genes

The expression of IL-8 greatly increased at 3 days after JFCC1 plasmid injection (Fig.5), which strongly supports the results of microarray.

Fig.3

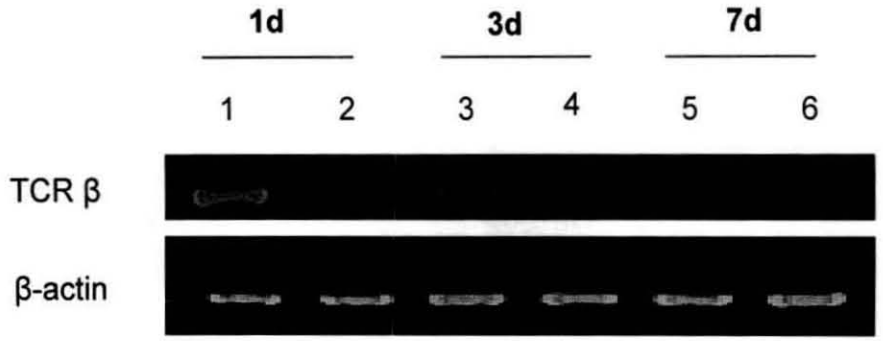


Fig.3 RT-PCR analysis of IL-8 inducible genes of Japanese flounder kidney cells in microarray. (A) TCR-β (B) β-actin. Expression of inducible genes on 1, 3 and 7 days after pCMV-IL-8 intramuscular injection (lanes 1, 3 and 5) and empty vector alone (lanes 2, 4 and 6).

Table: 3 Transcriptional profile of Japanese flounder kidney cells induced by IL-8

Group	Gene ID	Acc.No.	Gene Name	Expression		
				1 day	3 day	7 day
Humoral immunity	X43	AU260744	Ig M	**2.9**	1.1	0.8
	K49	AU260450	IgD	**2.1**	0.9	0.9
Cellular immunity	WH6-20F	AU050612	MHC classIA	**2.1**	ND	0.8
	O26	AU260532	T-cell receptor β	**2.1**	ND	ND
Other immune related genes	WE9-13F	AU050269	Heat shock factor binding protein 1	**2.1**	0.6	0.7
	WF12(1)	C81991	Hsc70	**2.1**	1	1
	OL10	BAA89039	Cytochrome oxidase subunit-3	**2**	1.1	ND
	B954	AU091153	Transcription factor BTF3	**2**	1.3	0.8
	LG3(6)	C23280,C23281	Apolipoprotein A-I	**0.3**	2	ND
	OL11	BAA89045	cytochrome-b	1.1	**0.4**	0.7
Metabolism related genes	WC11-17F	AU050462	Ribosomal protein L5	**2.3**	ND	ND
	WG5(2)	C82058	Proteasome subunit Z	**2.3**	ND	ND
	LB6(5)	C23180	Glycine decarboxylase	**2.1**	ND	ND
	WD8-15	AU050338	Nbr1	**2.1**	ND	ND
	B900	AU091133	Elongation factor1γ	**2**	1.1	ND
	OL47	Q9IA79	Probable Bax inhibotor-1	**2**	ND	ND
	V108	AU260703	Mast cell protease 3 (SMCP-3)	**2**	ND	1
	WG11(5)	C82261,C82262	Auxilin	**2**	ND	ND
	WC4(2)	C82026	DdGPK2	**2**	ND	ND
	V35	AU260661	L-lactate dehydrogenase M chain	**0.4**	ND	ND
	OL32	BAA89040	NADH dehydrogenase subunit-3	1	**0.5**	ND
	Z2	AU260861	Ribosomal protein L12	1	**0.5**	0.7
	L353	AU261112	L-plastin	0.9	**0.5**	0.8
	L152	AU260995	Low mol.Wt.Hsp30B	1	**0.5**	ND
	L217	AU261026	Syntaxin binding protein 3	0.9	**0.5**	0.7
	Y134	AU260848	Heparin	0.9	**0.4**	0.7
	L72	AU260937	Limulin	1	**0.4**	ND
	Y77	AU260807	Elongation factor1-α	1	**0.4**	ND
	Y45	AU260781	Ubiquitin A-52 residue ribosomal protein	0.8	**0.4**	0.7
	L211	AU261024	26S proteasome regulatory chain 12	1	**0.4**	ND
	Y50	AU260784	Human platelet profilin I	0.9	**0.4**	0.7
Unknown genes	HC5(1)	C23494,C23495	unknown	**2.9**	ND	ND
	WG6-8R	AU050069	unknown	**2.7**	ND	ND
	jfk0017/f	AU260427	unknown	**2.5**	ND	ND
	Y63	AU260794	unknown	**2.4**	ND	ND
	HC6(1)	C23496,C23497	unknown	**2.4**	ND	ND
	WE3(5)	C82254	unknown	**2.3**	1.1	0.7
	B1181	AU091252	unknown	**2.3**	ND	ND
	WH7-8R	AU050073	unknown	**2.2**	1.1	0.9
	LB9(8)	C23335	unknown	**2.2**	ND	ND
	B509	AU090975	unknown	**2.1**	ND	ND
	WD9-16F	AU050397	unknown	**2**	ND	ND
	L51	AU260920	unknown	**2**	0.9	0.7
	L145	AU260990	unknown	0.8	**0.5**	0.7
	B886	AU091124	unknown	1	**0.5**	0.7

Table.4 summary of microarray-based analysis of IL-8 inducible Japanese flounder kidney cells.

Class of genes			Number
Total number of genes analyzed			871
Known genes			500
Unknown genes			371
Days after pCMV-IL8 plasmid injection	1day	3day	7day
Up-regulated genes			
Known	18	1	0
Unknown	12	0	0
Total	30	1	0
Down-regulated genes			
Known	2	11	0
Unknown	0	2	0
Total	2	13	0
Total number of differentially expressed genes	32	14	0
Percentage(%) of differentially expressed genes	3.7	1.6	0

Table.6 summary of microarray-based analysis of JFCC1 inducible Japanese flounder kidney cells.

Class of genes			Number
Total number of genes analyzed			871
Known genes			500
Unknown genes			371
Days after pCMV-JFCC1 plasmid injection	1day	3day	7day
Up-regulated genes			
Known	8	27	6
Unknown	6	6	2
Total	14	33	8
Down-regulated genes			
Known	1	1	1
Unknown	1	2	1
Total	2	3	2
Total number of differentially expressed genes	16	36	10
Percentage(%) of differentially expressed genes	1.8	4.1	1.1

Table.5 Transcriptional profile of JFCC1 induced Japanese flounder kidney cells.

Group	Gene ID	Gene acc.No.	Gene name	Expression		
				1 day	3 day	7 day
Innate immunity	WD5(5)	C82250,C82251	TNFR 1	**0.5**	0.8	ND
	B1153	AU091230	CXC chemokine vIL8	1	**2.1**	ND
	cc chemoR A	Not reported	CC chemokine receptor A	1	**2**	0.9
	LB3(10)	C23436	complement component C3	**3.3**	ND	ND
	WB2-15F, -15	AU050313	unknown, lysozyme II	1	**2.1**	1
Humoral immunity	X43	AU260766,AU26097(	IgM	1	**2.1**	1.1
Cellular immunity	Y15	AU260758	Beta-2 microglobulin	1.2	**2**	1.4
Other immune related genes	B97	AU090725	Lymphocyte antigen LY-6G.1	0.9	**2.1**	1
	B866	AU091110	Amyloid protein AA	1.3	17	65
	WB2(4)	C82161	translational regulatory factor NAC/1.9.2	1.1	**2.4**	1.5
	L174	AU261008	dnaK-type molecular chaperone	1.1	**2.4**	1.4
	WG2-11R	AU050193	(Apoptosis-related protein TFAR15)	0.8	***1.3***	**2.3**
	LG3(6)	C23280,C23281	apolipoprotein A-I	**3.9**	0.6	**0.3**
	OL59	BAB83525	TBT-blinding protein	**3.8**	ND	ND
	LA8(1)	C23001	transferrin	**3.4**	0.7	0.4
	L201	AU261020	KIAA1532 protein	1	**2.3**	1.3
	LD7(2)	C23063	apolipoprotein E	1.2	**2.2**	1
Metabolism related genes	LA12(5)	C23175	prechymotrypsinogen	**3.1**	ND	ND
	LF1(4)	C23157	trypsin	**2.4**	ND	ND
	LE2(6)	C23265	fibrinogen alpha subunit	**2.3**	ND	ND
	OL30	BAA89033	NADH dehydrogenase subunit-1	**2**	1.1	1
	L317	AU261082	Ketohexokinase	1.1	**2.4**	1.4
	Y37	AU260775	(AK005147) putative	1	**2.3**	1.1
	B314	AU090867	Hypothetical 18K protein	1.1	**2.3**	1.5
	Y80	AU260810	mRNA for ribosomal protein S9	1.1	**2.3**	1
	Y3	AU260750	60S ribosomal protein L27A or L22	1.2	**2.3**	1.2
	WC5-14R	AU050279	cysteine-rich intestinal protein	1.2	**2.2**	1
	L123	AU260975	Gene HMG-T2 protein	1.1	**2.2**	1.2
	L394	AU261132	40S ribosomal protein S15A	1.6	**2.2**	2.5
	K29	AU260434	40S ribosomal protein S3	1.4	**2.2**	1.2
	LB9(1)	C23011	ribosomal protein S3	1.4	**2.1**	1.3
	LH8(1)	C23050	28S ribosomal RNA	0.9	**2.1**	1.4
	L211	AU261024	26S proteasome regulatory chain	0.9	**2**	1.4
	V35	AU260661	L-lactate dehydrogenase M chain	0.8	**2**	1.3
	WE5-8R	AU050053	myosin-IA	1.3	**2**	1.3
	B885	AU091123	60S ribosomal protein L21	0.8	**2**	ND
	Y137	AU260851	40S ribosomal protein 20S protein	1.2	**2**	1.1
	Y126	AU260840	Histone H1	0.9	**0.2**	0.8
	B710	AU091037	MC152R	0.7	1.9	**4.1**
	WE11(1)	C81980,C81981	ribosomal protein S17	0.9	1.3	**2.7**
	WB7-12F, -12	AU050220	dopamin receptor D1B, nk	0.8	1.1	**2.2**
Unknown genes	LG2(10)	C23474	uk	**4.2**	ND	ND
	LD4(5)	C23196, C23197	uk	**4.1**	ND	ND
	LB3(7)	C23294	uk	**3.6**	ND	ND
	LD10(1)	C23023	uk	**3.2**	ND	ND
	LE10(4)	C23155, C23156	uk	**2.4**	ND	ND
	LE11(9)	C23402	uk	**2.4**	ND	ND
	K40	AU260442	uk	**0.4**	ND	ND
	B117	AU090736	uk	ND	**5.7**	ND
	WG5-19F	AU050560	uk	0.9	**2.4**	ND
	L268	AU261055	uk	1.2	**2.3**	1.4
	L274	AU261058	uk	1.5	**2.2**	1.3
	L168	AU261005	uk	1	**2.1**	1.1
	B619	AU091005	uk	1	**2.1**	1.4
	LG10(5)	C23225, C23226	uk	0.7	**0.4**	ND
	LB3(8)	C23331	uk	0.9	**0.2**	ND
	Z23	AU260878	uk	1	1.6	**2.8**
	LE3(5)	C23206, C23207	uk	0.6	1.9	**2.1**
	LC4(7)	C23298	uk	ND	ND	**0.3**

Fig.4 Expression pattern of IL-8 inducible genes

Fig.5

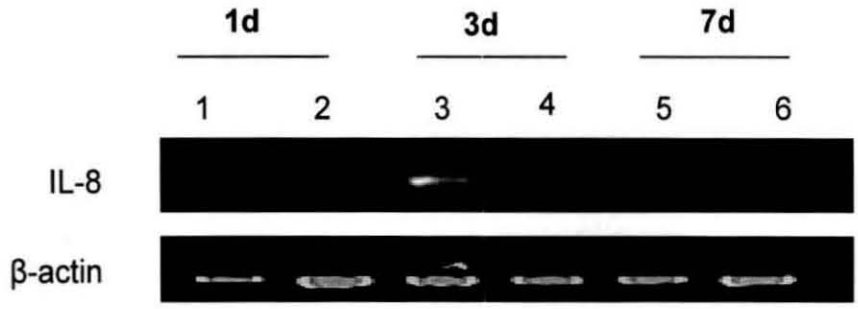


Fig.5 RT-PCR analysis of JFCC1 inducible genes of Japanese flounder kidney cells. (A) IL-8 (ID name:B1153), (B) β-actin . Expression of inducible genes on 1, 3 and 7 days after plasmid injection with pCMV-JFCC1 (lanes 1, 3 and 5) and empty vector alone (lanes 2, 4 and 6)

Fig. 6 Expression pattern of JFCC1 inducible genes

OL30 NADH dehydrogenase subunit-1
LG3(6) apolipoprotein A-I
LA8(1) transferrin
LE2(6) fibrinogen alpha subunit
LD4(5) Unknown
L174 dnaK-type molecular chaperone
L317 Ketohexokinase
WB2(4) translational regulatory factor NAC/1.9.2
B314 Hypothetical 18K protein
Y15 Beta-2 microglobulin
LA12(5) prechymotrypsinogen
LD10(1) Unknown
L268 Unknown
K29 40S ribosomal protein S3
LB9(1) ribosomal protein S3
LF1(4) trypsin
OL59 TBT-binding protein
LB3(10) complement component C3
L274 Unknown
LE11(9) Unknown
LE10(4) Unknown
LG2(10) Unknown
WE5-8R myosin-IA
Y80 mRNA for ribosomal protein S9
WC5-14R cysteine-rich intestinal protein
LD7(2) apolipoprotein E
LB3(7) Unknown
L123 Gene HMG-T2 protein
Y3 60S ribosomal protein L27A or L22
Y137 40S ribosomal protein 20S protein
WB2-15F, -15R unknown, lysozyme II
B1153 CXC chemokine vIL8
cc chemoR A CC chemokine receptor A
B117 Unknown
B97 Lymphocyte antigen LY-6G.1
WG5-19F Unknown
Y37 AK005147 putative
X43 IgM
L168 Unknown
LH8(1) 28S ribosomal RNA
L211 26S proteasome regulatory chain
L201 KIAA1532 protein
B619 Unknown
V35 L-lactate dehydrogenase M chain
B885 60S ribosomal protein L21
L394 40S ribosomal protein S15A
B866 Amyloid protein AA
Z23 Unknown
WG2-11R Apoptosis-related protein TFAR15
B710 MC152R
K40 Unknown
WE11(1) ribosomal protein S17
WB7-12F, -12R dopamin receptor D1B, nk
LE3(5) Unknown
WD5(5) TNFR 1
LC4(7) Unknown
Y126 Histone H1
LB3(8) Unknown
LG10(5) Unknown

3.4. DISCUSSION

Applications of DNA vaccines in aquaculture have been reported.[15] The use of expression plasmids for certain cytokines or co-stimulatory factors enhanced the immune responses induced with a DNA vaccine.[16-17] In this study, Japanese flounder fish vaccinated with 0.1 μg of pCMV-VHS g vaccine encoding the glycoprotein gene showed significant protection against VHS virus (52% survival). Co-administration of IL-1β, IL-8 and JFCC1 adjuvants with pCMV-VHS g vaccine significantly enhanced the survival rates of fish compared with fish given the pCMV-VHS g alone (Table.2).

We previously showed that the pCMV-VHS g vaccine had high protection efficiency, giving relative percentage survival values (RPS) of 93% with 10 μg of plasmid DNA.[3] However, a sub-optimal dose of vaccine antigen co-injected with cytokines proved valuable in assessing the adjuvant effects of cytokine genes.[18] In this report, a suboptimal dose of 0.1 μg of pCMV-VHS g vaccine was chosen in order to maximize the effect produced by co-injection of IL-1β, IL-8 and JFCC1 cytokine genes (10 μg). Although the results depended on the DNA dose and genetic background of the host, they indicate that an adjuvant effect is elicited by co-injection of IL-1 β, IL-8 and JFCC1 with pCMV-VHS g vaccine (Fig.1 & Table.2). Co-injection of these genes (10 μg) with 1 μg of pCMV-VHS g did not result in significantly higher survival rates than were obtained with DNA vaccine alone (Fig.2 & Table.2). Injection of the cytokine genes in the absence of the pCMV-VHS g did not elicit similar responses, indicating that the cytokines were acting as adjuvants rather than immunogens (Fig.1 & Table.2). These results agreed with the results of previous study [18] in which different cytokine genes were

used as adjuvants and immunogens to develop a vaccine against a protozoan infection in chickens.

The expression and immune responses following the injection of plasmid DNA containing the IL-1β [19] and a novel CC chemokine molecule [20] with the CMV promoter sequence in fishes have been reported. Fish injected with the CAT gene under the control of the CMV promoter showed high CAT expression.[21] In a microarray analysis of Japanese flounder kidney cells,[6] we showed that genes up-regulated by IL-1β were most strongly expressed on 1 day followed by 3 day and 7 day after plasmid injection. In the present study, we found that up-regulation of genes induced by IL-8 homologue in Japanese flounder kidney cells was highest on day 1 and that up-regulation of genes induced by JFCC1 was highest on day 3. This indicates that production of pro-inflammatory proteins such as IL-1β, IL-8 and JFCC1 was higher in the early stages after immunization. Other groups[19-20] also reported higher production of pro-inflammatory proteins in fishes in the early stages after immunization with pCMV-IL1β and a novel pCMV- CC chemokine.

IL-8 has been reported to attract T cells in vitro and in vivo.[22] Our microarray results of IL-8 induced kidney cells, up-regulated specific immune-related molecules such as MHC class IA and T cell receptor β suggesting that IL-8 is an activator of T cells. MIP-1α is a more potent chemoattractant with a broader specificity, attracting B cells and cytotoxic T cells as well as CD4+ T cells.[23-24] Our microarray results showed that JFCC1(MIP-1α), induced innate, humoral and cellular immunity-related molecules such as IL-8, CC chemokine receptor A, complement component C3, Ig M and β2-microglobulin. This observation suggests that the function of fish JFCC1 is similar to the

function of its mammalian homologues. IL-1β plasmid injection also induced humoral and cellular immunity molecules in Japanese flounder kidney cells.[6] The specific modulation of humoral and cellular immunity is the main basis for the prolonged immunity in DNA and adjuvant immunizations.[16] The up-regulation of both humoral and cellular immunity molecules in response to cytokine plasmid injection suggests that microarrays can be used as a reliable tool to develop effective vaccines.

A number of functionally unknown genes were also up-regulated, especially at 1 and 3 days after the injection of plasmid-encoded chemokines. The observed up-regulation of such genes during the early stages of post-plasmid injection suggests that they are involved in non-specific immunity in fish or that they trigger the expression of specific cellular and humoral responses. A potential area of research is to characterize some of the up-regulated unknown genes. This would identify novel immune-related genes.

The results of this study clearly document the role of fish cytokines as genetic adjuvants and still further studies are needed especially in the quantification of dosage and duration of immunity to develop effective vaccines using these cytokine genes.

ACKNOWLEDGEMENTS

This study was supported in part by Grants-in-Aid for Scientific Research (S) from the Ministry of Education, Science, Sports and Culture of Japan.

3.5. REFERENCES

1. Mortensen HF, Heuer OE, Lorenzen N, Otte L, Olesen NJ. Isolation of viral haemorrhagic septicemia virus (VHSV) from wild marine fish species in the Baltic Sea, Kattegat, Skagerrak and the North Sea. *Virus Res.* 1999; **63:** 95-106
2. Takano R, Mori K, Nishizawa T, Arimoto M, Muroga K. Isolation of viruses from wild Japanese flounder *Paralichthys olivaceus*. *Fish Pathol.* 2001; **36:** 153-160.
3. Byon JY, Ohira T, Hirono I, Aoki T. Use of a cDNA microarray to study immunity against viral haemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. *Fish Shellfish Immunol.* 2005; **18:** 135-147
4. Egan MA, Zimra R. The use of cytokines and chemokines as genetic adjuvants for plasmid DNA vaccines. *Clinical Applied Immunol. Rev.* 2002; **2:** 255-287
5. Secombes CJ, Zou J, Laing K, Daniels GD, Cunningham C. Cytokine genes in fish. *Aquaculture* 1999; **172:** 93-102
6. Emmadi D, Iwahori A, Hirono I, Aoki T. cDNA microarray analysis of IL-1β induced Japanese flounder, *Paralichthys olivaceus* kidney cells. *Fisheries Science* 2005; **71**(in Press)
7. Lee EY, Park HH, Kim YT, Choi TJ. Cloning and sequence analysis of the interleukin-8 gene from flounder (*Paralichthys olivaceus*). *Gene* 2001; **274:** 237-243
8. Khattiya R, Hirono I, Aoki T. Molecular cloning, gene structure and expression of two CC chemokines from Japanese flounder, *Paralichthys olivaceus. Fisheries Science* 2003; **69:** 1065-1074
9. Kurobe T, Yasuike M, Kimura T, Hirono I, Aoki T. Expression profiling of immune-

related genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. *Dev.Comp.Immunol.* 2005 (in Press)

10. Dhiman N, Bonilla R, O'Kane JD, Poland GA. Gene expression microarrays: a 21st century tool for directed vaccine design. *Vaccine* 2003; **21:** 22-30
11. Nishizawa T, Lida H, Takano R, Isshiki T, Nakajima K, Moroga K. Genetic relatedness among Japanese, American and European isolates of viral hemorrahagic septicemia virus (VHSV) based on partial G and P genes. *Dis. Aqua. Organisms* 2002; **48:** 143-148
12. Kasai H, Yoshimizu M. Establishment of two Japanese flounder embryo cell lines. *Bulletin Fish Science*, Hokkaido University 2001; **52:** 67-70 (in Japanese)
13. Sambrook J, Russel DW. Molecular Cloning, A Laboratory Manual, 3rd ed. New York: Cold Spring Harbour Laboratory Press; 2001. p. 1.65-1.74
14. Amend D. Potency testing of fish vaccines. *Dev. Biol. Standardization* 1981; **49:** 447-54
15. Heppel J, Davis HL. Application of DNA vaccine technology to aquaculture. *Adv. Drug Delivery Rev.* 2000; **43:** 29-43
16. Kim JJ, Simbiri KA, Sin JJ, Dang K, Oh J, Dentchev T, Lee D, Nottingham KL, Chalian AA, McCallus D, Ciccarelli R, Agadjanyan MG, Weiner DB. Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J. Interferon Cytokine Res.* 1999; **19:** 77-84
17. Kim JJ, Yang JS, Vancott TC, Lee DJ, Manson KH, Wyand MS, Boyer JD, Ugen KE, Weiner DB. Modulation of antigen-specific humoral responses in Rhesus Macaques by using cytokine cDNAs as DNA vaccine adjuvants. *J. Virol.*2000; **74:**

3427-3429

18. Min W, Lillehoj HS, Burnside J, Weining KC, Staeheli P, Zhu JJ. Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. *Vaccine* 2002; **20:** 267-74
19. Kono T, Fujiki K, Nakao M, Yano T, Endo M, Sakai M. The immune responses of common carp, *Cyprinus carpio L.*, injected with carp interleukin-1β gene. *J. Interferon Cytokine Res.* 2002; **22:** 413-419
20. Kono T, Kusuda R, Kawahara E, Sakai M. The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder, *Paralichthys olivaceus*. *Vaccine* 2003; **21:** 446-457
21. Tucker C, Endo M, Hirono I, Aoki T. Assessment of DNA vaccine potential for juvenile Japanese flounder Paralichthys olivaceus, through the introduction of reporter genes by particle bombardment and histopathology. *Vaccine* 2001; **19:** 801-09
22. Ward GS, Westwick J. Chemokines: understanding their role in T-lymphocyte biology. *Biochem .J.* 1998; **333:** 457-470
23. Schall TJ, Bacon K, Camp RDR, Kaspari JW, Goeddel DV. Human macrophage inflammatory protein α (MIP-1α) and MIP-1β chemokines attract distinct populations of lymphocytes. *J. Exp. Med.* 1993; **177:** 1821-1825
24. Lu Y, Xin KQ, Hamajima K, Tsuji T, Aoki I, Yang J, Sasaki S, Fukushima J, Yoshimura T, Toda S, Okada E, Okuda K. Macrophage inflammatory protein-1α (MIP-1α) expression plasmid enhances DNA vaccine-induced immune response against HIV-1. *Clin. Exp. Immunol.* 1999; **115:** 335-341

Chapter 4

Cloning, characterization and expression analysis of interferon-induced protien-10 (IP-10) in Japanese flounder (*Paralichthys olivaceus*)

4.1. ABSTRACT

Interferon-inducible protein (IP-10) was identified for the first time in fish from Japanese flounder. The gene was obtained from brain cells stimulated with poly I:C. The cDNA consists of 677 bp with a 27 bp 5' UTR, a 336 open reading frame encoding a 111 amino acid peptide and a 314 bp 3' UTR. The gene has four cysteine residues, which are conserved, with first two cysteines separated with histidine. Homology and Phylogenetic analysis revealed that the gene was found to be closer to human IP-10. Identities were significantly low to the SCYBA gene cloned from zebrafish , eventhough it shared the same cluster. The gene was transcribed from an early time point by stimulation with LPS and poly I:C.

***Keywords*: fish; cytokines; innate immunity; inflammation; mitogens; RT-PCR**

4.2. INTRODUCTION

Chemokines are a super family of small related protein molecules that are secreted by a variety of cells and their ability to recruit a wide range of immune cells to the sites of infection and disease. These molecules act as chemo-attractants causing an influx of neutrophils, monocytes, T-cells and basophils in humans. Chemokines are classified into four groups: CXC(α), CC(β), C(γ) and CX3C(δ) based on the arrangement of first two cystein residues within the protein[1]. In CXC chemokines the two N-terminal cysteines are separated by one intervening aminoacid.

Chemokines in teleosts have been broadly investigated during the past few years. Most fish chemokines have been described from molecular cloning of their cDNAs and from deducing their aminoacid sequences. Interferon-inducible protein-10 (IP-10) is member of a CXC chemokine super family of proinflammatory cytokines having chemotactic, mitogenic and immuno-modulatory activities[2]. It was induced by IFN-γ and LPS [3]. IP-10 is constitutively expressed in lymphoid organs[4]. IP-10 is also expressed in many inflammatory diseases[5].

In the present study, we describe the isolation, characterization and expression analysis of a cDNA transcript encoding IP-10 for the first time in teleosts.

4.3. MATERIALS AND METHODS

4.3.1. Construction of cDNA library

Total RNA was isolated from Japanese flounder brain cells stimulated with poly IC at 1 hr and 3 hr. The mRNA was purified using a quick prep micro mRNA kit (Amersham Biosciences, U.S.A) following the manufacturer's instructions. cDNA

synthesis was performed using a SMART cDNA library construction kit (BD Biosciences, U.S.A), in a pTriplEx2 cloning vector (BD Biosciences, U.S.A) following the manufacturer's instructions.

4.3.2. Cloning of IP-10

A partial cDNA clone of IP-10 obtained from the brain cDNA library, was used as a DNA probe to screen a full length cDNA of IP-10. The hybridization was conducted as previously reported [6]. The plasmid DNA was extracted by alkaline lysis method[7]. The cDNA clones nucleotide sequences are determined on an automated DNA sequencer LC4200 (Li-cor, U.S.A). The nucleotide and deduced amino acid sequences were analyzed by using GENETYX windows version 8.0 (SDC software Development, Japan). Each obtained cDNA sequence was compared with sequences deposited in DDBJ/EMBL/GenBank using the BLASTX and BLASTP programs [National Center for Biotechnology Information (NCBI), available at http://www.ncbi.nlm.nih.gov/BLAST]. The signal IP program was used to predict the cleavage site between the signal peptide and mature IP-10 (available at http://www.cbs.dtu.dk/services/signalP/)[8].

4.3.3. Alignment analysis and Phylogenetic tree construction

The amino acid and nucleotide sequences of IP-10 genes were retrieved from NCBI databases. Multiple alignments of IP-10 genes were carried out using clustal X program [9]. A phylogenetic tree of IP-10 genes based on amino acids was constructed with the neighbor-joining method using the PHYLIP program. The values supporting each node are derived from 100 resamplings.

4.3.4. IP-10 gene expression in flounder tissues

Total RNA was extracted from healthy Japanese flounder brain, eye, gill, head kidney, heart, intestine, liver, muscle, ovary, PBLs, skin, spleen, stomach and post kidney using Trizol Reagent (Invitrogen, USA). The first strand cDNA was synthesized using first strand cDNA synthesis kit with AMV reverse transcriptase following the manufacturer's instructions (Life Sciences, USA). One microliter of reverse-transcribed reaction from each tissue was used as a template for PCR amplification. The primers used in this experiment were listed in Table.1. A β-actin primer set [10] was used as a positive control. The PCR reaction was conducted with an initial denaturation step at 95 ^{0}C for 2 min, followed by 30 cycles of 95 ^{0}C for 30 s, 55 ^{0}C for 30 s, and 72 ^{0}C for 1 min. The reacted products were electrophoresed on a 2% agarose gel containing ethidium bromide (100 ng/ml).

4.3.5. IP-10 gene expression in vitro

Head kidney cells of Japanese flounder were stimulated by treatment with Con A (0.05 μg), PMA (0.1 μg/ml), LPS (0.5 mg/ml) and poly I:C (5.0 μg) at 1, 3 and 6 hr intervals.

Table 1 RT-PCR primers for IP-10 expression anlysis

Clone	Accession no.	Gene	Primer	C
B120	AU090737	β-actin (sense)	5'-TGATGAAGCCCAGAGCAAGA-3'	2
		β-actin (antisense)	5'-CTCCATGTCATCCCAGTTGG-3'	
IP-10	Not submitted	IP-10 (sense)	5'-CGAAGATGAAGCTGCAG-3'	
		IP-10 (antisense)	5'-ATAGCGATTCAGAAGTCCGG-3'	

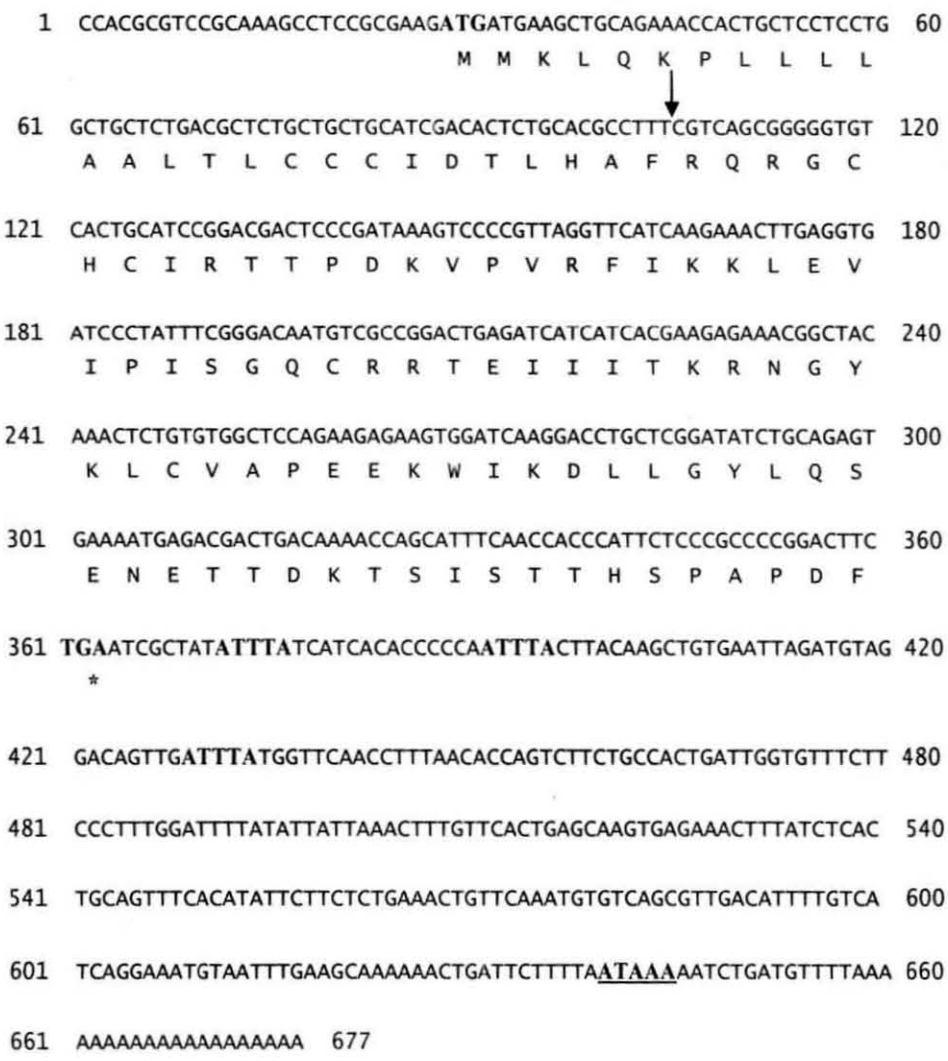
Total RNA was extracted by Trizol Reagent (Invitrogen, USA) following manufacturer's instructions. The first strand cDNA was synthesized using first strand cDNA synthesis kit

with AMV reverse transcriptase following the manufacturer's instructions (Life Sciences, USA). One microliter of reverse-transcribed reaction from each sample was used as a template for PCR amplification. The primers used in this experiment were listed in Table.1. A β-actin primer set[10] was used as a positive control. The PCR reaction was conducted with an initial denaturation step at 95 ^{0}C for 2 min, followed by 30 cycles of 95 ^{0}C for 30 s, 55 ^{0}C for 30 s, and 72 ^{0}C for 1 min. The reacted products were electrophoresed on a 2% agarose gel containing ethidium bromide (100 ng/ml).

4.4. RESULTS AND DISCUSSION

Chemokines are key components in the process of leukocyte recruitment in inflammatory sites. The interaction of various chemokines with their receptors on leukocytes allows activation of chemotaxis of neutrophils, eosinophils, lymphocytes, monocytes necessary for migration to the sites of evolving inflammation. We have identified interferon inducible protein-10 (IP-10) for the first time in teleosts from Japanese flounder brain cDNA library stimulated with Poly I:C. The identification and cloning of Japanese flounder CXC chemokine was based on the EST analysis. Poly I:C is known to trigger a wide range of cellular responses, including the production of cytokines and chemokines. The Japanese flounder cDNA consists of 677 bp with a 27 bp 5' UTR, a 336 open reading frame encoding a 111 amino acid peptide and a 314 bp 3' UTR (Fig. 1). The 3' UTR contains a single typical polyadenylation signal (AATAAA) and three instability motifs (ATTTA) responsible for rapid degradation of mRNA (Fig. 1), as is the case with other inflammatory molecules [11]. The presence of consensus sequence in other CXC chemokines in fishes [12]. The predicted cleavage site of the signal sequence to

Fig.1



1. Complete full length Japanese flounder IP-10 cDNA sequence. Start and stop codon, RNA instability motifs (ATTTA)in the 3' untranslated region are indicated in bold letters. The vertical arrow indicates the signal peptide cleavage site. The polyadenylation signal (AATAAA) is underlined. Stop codon is also given asterisk mark.

the mature protein is between phenyl alanine and Arginine. The mature peptide has four cysteine residues, which are conserved, with first two cysteines separated with histidine

(Fig.2). Japanese flounder IP-10 has all the four cysteines and in conserved positions as found in mammalian IP-10s. It is interesting to note that histidine separates the first two cysteines forming the CXC motif.

Interestingly, the ELR (Glutamate-leucine-arginine) motif, which is associated with specificity to neutrophil between the N-terminus and the first cysteine is absent in IP-10 (Fig.2). Japanese flounder IP-10 is longer than the human IP-10. The amino acid sequence of Japanese flounder draws highest identity (Fig. 2) to mouse IP-10 (47.3%) followed by human (44.1%), Macaque (42.8), sheep IP-10 (41.6%) and goat IP-10 (40.5%) when mature protein was compared (Table 2). A molecular Phylogenetic tree was constructed to further analyze the evolutionary relationship with known IP-10 chemokines. The phylogenetic analysis revealed that Japanese flounder IP-10 is closely related with zebrafish SCYBA, and human, macaque, sheep and goat IP-10s (Fig. 3). In unison to the percentage identity, the Japanese flounder IP-10 occupied a different clad in relation to the zebrafish SCYBA and was closely related to human IP-10. According to Najakshin et al. (1999) [13] early divergence of CXC type chemokine lead to three basic types: SDF, IL-8 and I-TAC. The CXC chemokines so far isolated in fish are homologues of human IL-8 like chemokines [14]. Japanese flounder IP-10 is also of IL-8 type. Japanese flounder IP-10 shows higher homology to mouse and human IP-10. IL-2 and interferons up-regulate the non-ELR CXC chemokines like IP-10, Mig and SDF-1. This suggests that,

Fig. 2

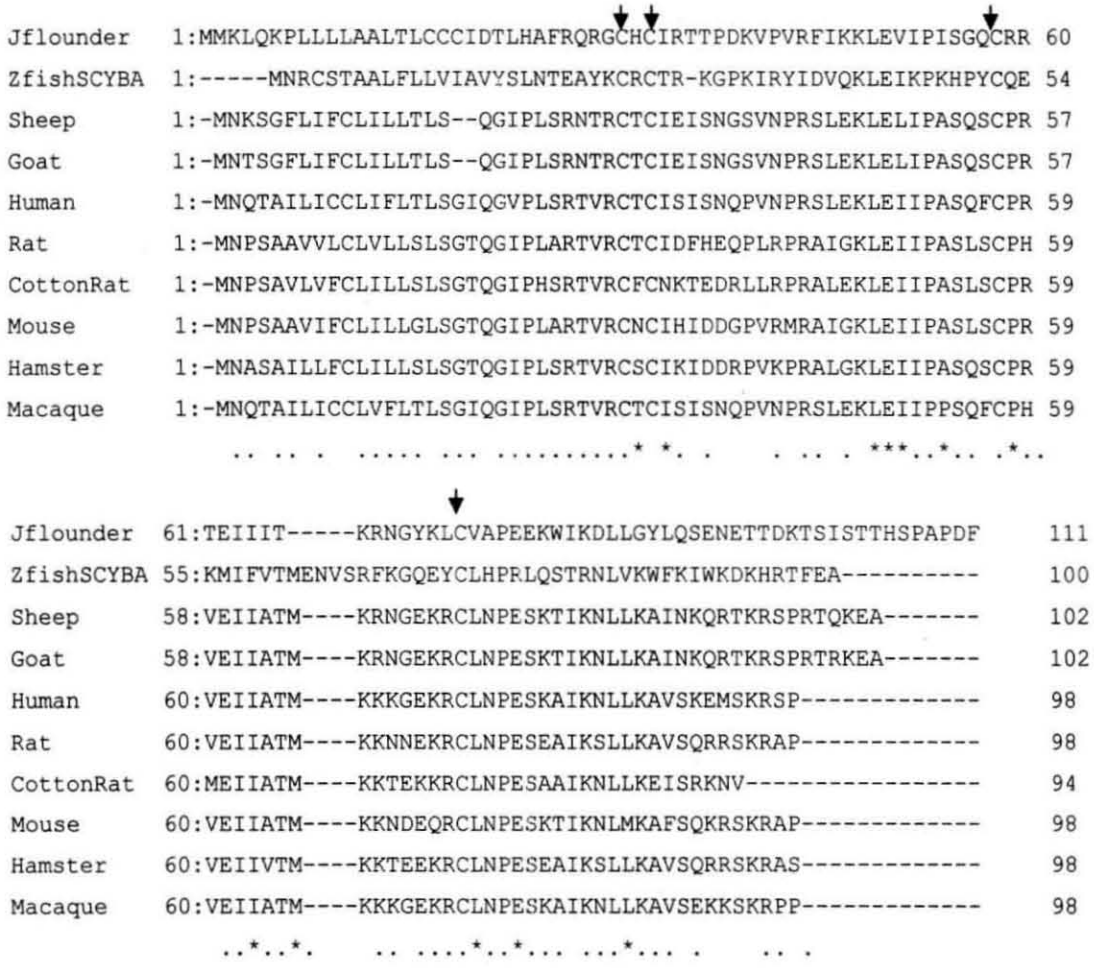


Fig. 2 Alignment of the predicted Japanese flounder IP-10 translation with other known IP-10s. Identical (*) and similar (.) residues identified by CLUSTAL are indicated. Triangles indicate the CXC motif.

Table 2 Amino acid percentage homology of Japanese flounder IP-10 with other known IP-10 genes

Species	Amino acid identity
Zebrafish	30.10%
Human	44.1
Sheep	41.6
Goat	40.5
Rat	38.7
Cotton rat	38.9
Mouse	47.3
Hamster	36.5
Macaque	42.8

the interferon and IL-2 genes may be present in fish but not yet cloned in fish. IP-10 and Mig have selectivity to T-cells that have been stimulated by IL-2 and interferon. This results in the recruitment of activated/effector T cells thereby, initiating the effector T cell immunity [15]. Furthermore, chemokines are known to exert their biological activities through G-protein coupled receptors of the target cells. In mammals, receptors of CXC and CC have been identified. The IP-10 has specificity to CXCR3, which is expressed on activated T cells and is important in selective recruitment of T cells during inflammation [16]. However functional studies have to be carried out to know the receptor specificity of Japanese flounder IP-10 and its role during inflammation.

A survey of IP-10 transcript expression in normal tissues was undertaken. IP-10 was detected in ovary, kidney, spleen, eye, skin and gill of healthy Japanese flounder. The IP-10 expression was not seen in the brain or heart (Fig. 4). These results confirming observations that constitutive expression of IP-10 in normal tissue is generally quite low

Fig. 3

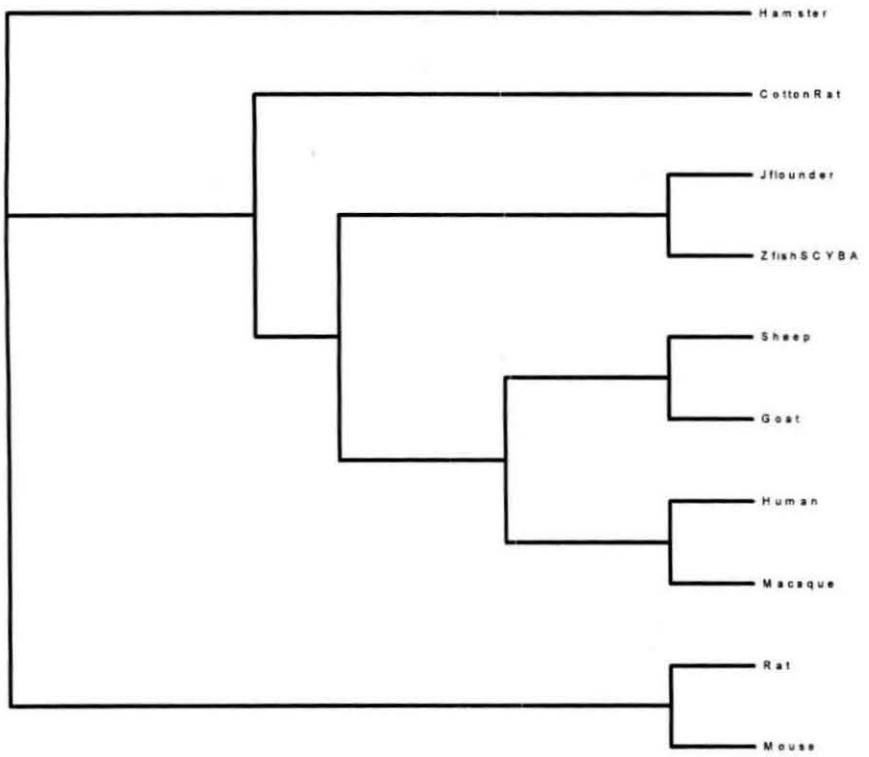


Fig. 3 Phylogenetic tree showing the relationship between the full-length Japanese flounder IP-10 amino acid sequence with other representative IP-10 sequences in different vertebrate groups. The tree was constructed by the 'neighbour-joining' method using the CLUSTAL and PHYLIP packages.

[17]. After poly I:C and LPS induction of kidney cells the IP-10 expression was transcribed at an higher level (Fig. 5). The poly I:C and LPS challenge at various time intervals indicated that the IP-10 is induced at an early time point and the message is strong in the

early period of induction. Although the physiological role of IP-10 remains to be determined, it probably participates in inflammatory processes. Furthermore, IP-10 has been shown to be a chemoattractant for monocytes and activated T cells[18]. The chemokines demonstrate protracted expression kinetics. The expression of the gene may reflect the importance in acute and chronic stresses in fish. At tissue level, the expression in all organs may be due to tissue macrophages present in the organs.

Fig. 4

1 Brain, 2 Eye, 3 Gill, 4 Head kidney, 5 Heart, 6 Intestine, 7 Liver, 8 Muscle, 9 Ovary, 10 PBLs, 11 Skin, 12 Spleen, 13 Stomach, 14 Post kidney

Fig. 4 Transcripts of IP-10 from different organs of Japanese flounder

Fig. 5

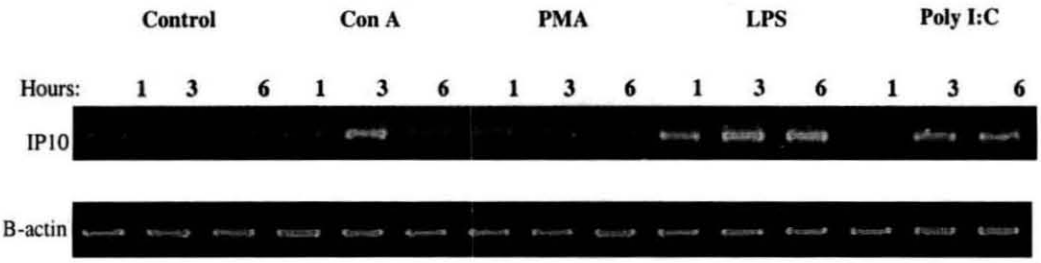


Fig. 5 Expression analysis of head kidney cells stimulated with ConA, PMA, LPS and poly I:C.

We have cloned cDNA of IP-10 in teleosts for the first time. The cDNA and expression data will provide a solid basis for conducting further functional studies of this gene.

ACKNOWLEDGEMENTS

The present study was supported in part by a Grant-in-Aid for Scientific Research (S) of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

4.5. REFERENCES

1. Rollins BJ. Chemokines. *Blood* 1997; **90:** 909-928.
2. Luster AD, Ravetch JV. Genomic organization of a gamma-interferon-inducible gene (IP-10) and identification of an interferon-inducible hypersensitive site. *Mol. Cell.Biol.* 1987; **7:** 3723-3731.
3. Luster AD, Leder P. IP-10, a CXC chemokine, elicits a potent thymus-dependent antitumor response *in vivo. J. Exp. Med.icine* 1993; **178:** 1057-1065.
4. Gattass CR, King LB, Luster AD, Ashwell JD. Constitutive expression of IP-10 in lymphoid organs and inducible expression in T cells and thymocytes. *J.Exp. Med.* 1994; **179:** 1373-1378
5. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-γ-inducible protein 10 (IP-10)-deficient mice reveal a role for IP-10 in effector T cell generation

and trafficking. *J.Immunol.* 2002; **167:** 3195-3204.

6. Hirono I, Nam BH, Kurobe T, Aoki T. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder, *Paralichthys olivaceus*. *J. Immunol.* 2000; **165:** 4423-4427.
7. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning : A Laboratory Manual, 3 rd ed. Cold Spring Harbor Laboratory Press, New York.
8. Nielsen H, Engelbrecht J, Brunak S, Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Prot. Engineering* 1997; **10:** 1-6.
9. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DJ. The clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality anlaysis tools. *Nucleic Acids Res.* 1997; **25:** 4876-4882.
10. Katagiri T, Hirono I, Aoki T. Idenification of a cDNA for medaka cytoskeletal β-actin and construction for the reverse transcriptase-polymerase chain reaction (RT-PCR) primers. *Fish. Sci.* 1997; **63:** 73-76.
11. Caput D, Beutler B, Hartog K, Thayer R, Brown-shimer S, Cerami A. Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. *Proc. Nat. Acad. Sci., USA* 1986; **83:** 1670-1674.
12. Savan R, Kono T, Aman A, Sakai M. Isolation and characterization of a novel CXC chemokine in common carp (*Cyprinus carpio L.*). *Mol. Immunol.* 2003; **39:** 829-834.
13. Najakshin AM, Mechetina LV, Alabyev BY, Taranin AV. Identification of an IL-8 homolog in lamprey (*Lampetra fluviatilis*): early evolutionary divergence of

chemokines. *Eur. J.Immunol.* 1999; **29**: 375-382.

14. Secombes CJ, Wang T, Hong S, Peddie S, Crampe M, Laing KJ, Cunningham C, Zou J. Cytokines and innate immunity in fish. *Dev. Comp. Immunol.* 2001; **25:**713.
15. Mackay CR. Chemokine receptors and T cell chemotaxis. *J.Exp. Med.* 1996; **184:** 799-802.
16. Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggiolini M, Moser B. Chemokine receptor specific for IP-10 and Mig: structure, function and expression in activated T-lymphocytes. *J. Exp. Med.* 1996; **184:** 963-969.
17. Narumi S, Wyner LM, Stoler MH, Tannenbaum CS, Hamilton TA. Tissue specific expression of murine IP-10 mRNA following systemic treatment with interferon-γ. *J.Leukocyte Biol.* 1992; **52:** 27-29.
18. Taub DD, Llyod AR, Conlon K, Wang JM, Ortaldo JR, Harada A, Matsushima K, Kelvin DJ, Oppenheim JJ. Recombinant human interferon-inducible protein-10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J.Exp. Med.* 1993; **177:** 1809-1814.

Chapter 5

Summary

Cytokines are low molecular weight soluble proteins secreted by the cells of the innate and adaptive immunity that mediate many of the functions of these cells. They are often pleotropic and redundant. In the present study we investigated the adjuvant effects of IL-1β, IL-8 homologue of CXC chemokine and JFCC1 which is a homologue of MIP-1α using VHSV as a model system. We also enumerated the transcriptional profiles of Japanese flounder kidney cells induced by IL-1β, IL-8 and JFCC1 to know how these genes influence the fish immune system as a whole.

In this study, IL-1β, IL-8 and JFCC1 full-length gene sequences were cloned in DNA vaccine expression vector driven by CMV promoter. In vitro transcription and translation was done to know whether the nucleotide sequences were translated into protein or not. Later the plasmids were extracted in a large scale and purified by ethidium bromide-cesium chloride ultra centrifugation. The adjuvants were injected into Japanese flounder fish along with VHS g vaccine. The fish were challenged with $1X10^3$ $TCID_{50}$ by intra/peritoneal injection. The RPS and cumulative mortalities were recorded. In the second part of study, IL-1β, IL-8, JFCC1 and empty vector expression plasmids were injected into Japanese flounder fish and the kidney tissue was extracted at 1 day, 3 day and 7 day post-intra/muscular injection. Total RNA was extracted and was converted into cDNA. The cDNA was labeled with Cy3 and Cy5 fluorescent substances. The labeled sample was hybridized with cDNA microarray chip. After washing the microarray slide was scanned with Genepix 4000b scanner. The results were analyzed by using computer.

In Chapter 2 we described cloning, characterization and expression of IL-1β and transcriptional profiling of Japanese flounder kidney cells induced by IL-1β. IL-1β cDNA found to consist of 1329 bp, encoded 247 amino acid residues. The expression of IL-1β was induced by treatment with ConA/PMA and LPS. The copy number of IL-1β mRNA was increased 30 fold after stimulation with ConA/PMA. Of 871 cDNA on a microarray, 10.7% of the genes were up-regulated or down-regulated by IL-1β at 1, 3 and 7 days post injection. The induced gene expression was highest on 1 day followed by 3 day and 7 day. Seven percent of known and 3.7% of unknown genes of the 871 tested genes were differentially expressed. Cytokine genes such as tumor necrosis factor, G-CSF and chemokine receptor A were induced in response to IL-1β. Cell surface antigens such as Ig M, MHC class I and CD20 receptor were up-regulated. Signal transduction genes such as Toll-like receptor 1 and SH3P2 were also up-regulated. The glucocorticoid receptor and cAMP early repressor were down regulated in our microarray analysis.

In chapter 3 we described adjuvant effects of IL-1β, IL-8 and JFCC1 against VHSV and also the analysis of the immune response of IL-8 and JFCC1 genes using cDNA microarrays. The adjuvants used in this study were able to provide protection against VHS virus and this lasted until 21 days after challenge. The adjuvants vaccine had high protective efficiency, giving relative percentage survival values of at least 70%. The defense mechanisms activated by two of the cytokines (IL-8, JFCC1) were further elucidated by microarray analysis. IL-8 up-regulated genes such as IL-8, CC chemokine receptor A, lysozyme, Ig M and β2-microglobulin.

In chapter 4 we described cloning, characterization and expression analysis of interferon-inducible gene-10. IP-10 is also a chemokine which can be used as adjuvant in

vaccine studies. In our phylogenetic analysis we found that it is closely related to human IP-10.

In conclusion, IL-1β, IL-8 and JFCC1 showed adjuvant effects in DNA vaccine against VHSV in our study. All these three genes are strong mediators of innate immunity, humoral immunity and cellular immunity mechanisms. All three genes induced inflammatory related genes. IL-1β alone exhibited the signal transduction inducer property. Still further studies are needed especially in the quantification of dosage and duration of immunity to assess long lasting adjuvant properties of these cytokine genes.